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CHEESE-MAKING BY FULL CONCENTRATION OF MILK WITH MEMBRANE FILTRATION AND EVAPORATION

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ACADEMIC DISSERTATION

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Abstract

Almost 40 years ago, a cheese-making process was described in which milk was concentrated to the final total solids content of cheese and no whey draining was made after coagulation. This full concentration (FC) process has since been used in soft cheese-making. However in semi-hard and hard cheese-making concentrated whey proteins have had negative effect on cheese flavor and texture and therefore the technique has not been used for these products. Development of filtration techniques has made it possible to fractionate milk components. Concentration of the major cheese components, fat and casein, is possible with microfiltration (MF). The aim of this study was to develop an FC cheese process using MF and evaporation steps.

Cheese-making with the FC process consists of whey protein removal and standardization of lactose and calcium contents. During whey protein removal plasmin (PL) was activated and its heat stability was increased. Protein hydrolysis by PL before cheese-making may reduce cheese yield and coagulation properties and therefore the FC process must be continuous. The calcium-protein ratio affects the final structure of cheese and can be standardized with acidification and filtration steps. During FC of milk, the viscosity of retentate increases and its processability decreases. It was found that acidification reduced the viscosity of retentate and slowed the increase of viscosity during concentration. This observation may be important for FC process development.

Secondary proteolysis of FC cheese was apparently at a low level, because no free amino acids (FAA) were found at the end of ripening. Added peptidase increased the FAA content in cheese and with enzyme addition it was possible to alter the ripening process. However, peptidase addition also changed lactose fermentation, and therefore the microbiological composition of LAB changed in cheese. The effects of $CaCl_2$ addition on FC cheese ripening were studied. It was found that $CaCl_2$ addition increased the growth of LAB, probably due to delayed lysis of LAB. It appears that standardization of calcium content is essential to control lyses caused by LAB, which affect cheese ripening.

Tiivistelmä

Noin 40 vuotta sitten esitettiin juuston valmistusprosessi, jossa maito konsentroitiin juuston lopulliseen kuiva-aineeseen eikä perinteistä heran erottelua juoksettamisen jälkeen tehty. Tätä täyskonsentrointimenetelmää (FC) on käytetty pehmeiden juustojen valmistuksessa, mutta puolikovia ja kovia juustoja valmistettaessa konsentroituneilla heraproteiineilla oli negatiivinen vaikutus juuston makuun ja rakenteeseen, eikä menetelmää ole käytetty kyseisten juustolaatujen valmistuksessa. Suodatusmenetelmien kehittymisen myötä maidon komponenttien fraktiointi tuli mahdolliseksi ja juuston peruskomponentit, rasva ja kaseiini voitiin konsentroida käyttämällä mikrosuodatusta. Tämän tutkimuksen tarkoituksena oli kehittää FC juuston valmistusprosessi hyödyntämällä suodatus- ja haihdutusvaihetta.

FC juuston valmistusprosessi sisältää heraproteiinien poistamis- sekä laktoosin ja kalsiumin vakiointivaiheet. Heraproteiinien poiston havaittiin aktivoivan plasmiinin (PL) ja parantavan sen lämpöstabiiliutta. Proteiinien hydrolysoituminen PL:n vaikutuksesta ennen juuston valmistusta voi laskea juustosaantoa ja heikentää juoksettumisominaisuuksia ja sen takia FC juuston valmistusprosessin tulee olla jatkuvatoiminen. Kalsiumin ja proteiinin suhde vaikuttaa lopullisen juuston rakenteeseen ja se voidaan vakioida hyödyntämällä hapatus- ja suodatusvaihetta. FC:n yhteydessä tiivisteen viskositeetti kasvaa ja prosessoitavuus heikkenee. Hapattamisen havaittiin laskevan tiivisteen viskositeettia ja hidastavan sen kasvua konsentroinnin aikana. Tämä havainto saattaa olla yksi tärkeimmistä valmistettaessa FC juustoa.

Sekundääriproteolyysi oli hyvin alhainen FC juustossa, koska vapaita aminohappoja (FAA) ei havaittu edes kypsymisen lopulla. Peptidaasi-entsyymiä lisäämällä pystyttiin kasvattamaan FAA määrää juustossa, mutta samalla havaittiin muutosta laktoosin fermentoitumisessa, joka viittaa mikrobipopulaation muuttumiseen. Lisätyn $CaCl_2$:n vaikutusta FC juuston kypsymiseen tutkittiin. $CaCl_2$:n havaittiin kasvattavan maitohappobakteerien (LAB) pitoisuutta ja vähentävän niiden lyysaantumista. Tuloksen perusteella voidaan olettaa että kalsiumpitoisuuden vakioinnilla voidaan kontrolloida LAB elävyyttä, mikä vaikuttaa juuston kypsymiseen.

Preface

The experimental part of this thesis was carried out during 2010-2012. Experiments were executed at Pilot Dairy of University of Helsinki, Pilot Cheese plan of Valio Ltd at Lapinlahti, Special product plan of Valio Ltd at Lapinlahti and Research and Development Center of Valio Ltd at Helsinki.

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Helsinki, 2012

List of Publications

This thesis is based on an overview and references to the following publications indicated in the text by Roman numerals.

I. Aaltonen, T., & Ollikainen, P. (2011). Effect of microfiltration on plasmin activity. *International Dairy Journal*, 21, 193-197.

II. Aaltonen, T. (2012). Standardization of the calcium content of whey protein-free milk concentrate. *International Journal of Dairy Technology*, 65, 178-182.

III. Aaltonen, T. (2012). Effect of acidification of whey protein-free precheese retentate on viscosity increase at different concentrations. *LWT – Food Science and Technology*, 47, 8-12.

IV. Aaltonen, T., & Huumonen, I. (2012). Effect of peptidase addition on ripening of cheese made from full concentrated milk retentate. Under review.

V. Aaltonen, T. (2012). Effect of $CaCl_2$ addition on lactic acid bacteria viability in cheese made from full concentrated milk retentate. Under review.

Author's contribution

I. Terhi Aaltonen planned and executed the experiments, analyzed the results and wrote the article. Pia Ollikainen introduced plasmin activity analysis for Valio Ltd and confirmed plasmin activity results.

II. Terhi Aaltonen planned and executed the experiments, analyzed the results and wrote the article.

III. Terhi Aaltonen planned and executed the experiments, analyzed the results and wrote the article.

IV. Terhi Aaltonen planned and executed the experiments with Ilkka Huumonen. Terhi Aaltonen analyzed the results and wrote the article.

V. Terhi Aaltonen planned and executed the experiments, analyzed the results and wrote the article.

List of Abbreviations

α-LA	α-lactalbumin
β-LG	β-lactoglobulin
γ	shear rate
ΔP	pressure difference
η	viscosity
η_o	consistency coefficient
$\eta/\ \eta_{ref}$	relative viscosity
ø	volume fraction (volume of component X concentration of component)
$ø_{max}$	maximum volume fraction
BSA	bovine serum albumin
C_F	concentration of component in feed
C_P	concentration of component in permeate
C_R	concentration of component in retentate
cfu	colony-forming unit
CF	concentration factor
CN	casein nitrogen
DF	diafiltration
FAA	free amino acid
FC	full concentrated
GDL	glucono-δ-lactone
G6PDH	glucose-6-phosphatase dehydrogenase
I	concentration of inhibitor
J	flux
K'_I	inhibitory constant for uncompetitive inhibition
K_I	inhibitory constant for competitive inhibition
K_m	substrate concentration at half-V_{Max}
LAB	lactic acid bacteria
LDH	lactate dehydrogenase
MF	microfiltration
n	flow behavior index

NF	nanofiltration
NPN	non-protein nitrogen
Met	methionin
PG	plasminogen
Pep-X	X-prolyl-dipeptidase
Phe	phenylalanin
PL	plasmin
PPDA	post-proline dipeptidyl amino peptidase
RP-HPLC	reversed-phase high-performance liquid chromatographic
R	resistance factor
RO	reverse osmosis
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SW	spiral wound
TN	total nitrogen
TS	total solids
UF	ultrafiltration
UHT	ultra high -temperature sterilization
V	initial velocity
V_F	volume of feed
V_{Max}	maximum velocity
V_R	volume of retentate
WP	whey protein

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1. Introduction

Cheese-making began during the Agricultural revolution 6 000 - 8 000 years ago. It is commonly believed that the first cheese was produced accidentally when lactic acid bacteria (LAB) grew in nutrient rich milk. As a consequence of lactose fermentation by LAB, the pH decreased and milk coagulated. When the coagulated milk was broken, the whey and cheese curd were formed (Fox & McSweeney, 1998). For a long time whey was the main product because it was a fresh and highly nutritive drink. Salting of cheese curd increased its shelf life and made cheese a usable food. Development of rennet-coagulated cheese improved cheese texture and made it a more popular product (Fox et al., 2004). Today more than 14.7 billion kg of cheese is made worldwide and approximately 33% of produced milk is used in cheese-making (USDA, 2011)

As earlier, cheese-making is still a concentration process in which fat and caseins are concentrated and most of whey proteins, lactose and salts are removed with whey. Approximately 10 kg of milk is needed for the production of 1 kg of cheese, and at the same time 9 kg of whey is formed (Fox & McSweeney, 1998). This high amount of starting material with different water additions makes the energy efficiency of cheese-making rather low. Seasonal changes in milk composition and quality also affect the quality of the final cheese product (Fox et al., 2004). One of the main problems in cheese-making is the high amount of byproduct whey compared to the amount of cheese produced. Whey is a valuable nutrient-rich fraction and has been used as an ingredient for infant formulas and dietetic and health foods (de Wit, 1998). However, cheese-making affects the quality of the final whey product which must be taken into account in the development of cheese-making processes.

The development of filtration techniques made it possible to concentrate and fractionate components of milk. Almost 40 years ago, a method was described in which ultrafiltration (UF) was used to concentrate milk 5-fold or more and this concentrate was used in cheese-making without curd cutting and whey draining (Maubois & Mocquot, 1974). However, this full concentrated (FC) cheese-making method has been commercially used only in soft cheese-making. In semi-hard and hard cheeses, whey proteins of UF-retentate reduce proteolysis during ripening and affect negatively the texture of cheese (Horton, 1997). As a consequence FC cheese-making has not been used for production of semi-hard and hard cheeses (Bastian et al., 1993; Harper et al., 1989; Lelievre et al., 1990).

Development of filtration techniques, and especially of microfiltration (MF) with 0.05-0.3 µm cut-off membranes, has enabled the removal of whey proteins from the retentate and concentration of the main components of cheese, i.e. fat and casein (St-Gelais et al., 1995; Brandsma & Rizvi, 1999). Cheese milk concentration by MF has yielded some major advantages in cheese-making, including reduced rennet addition, increased proteolysis and better cheese-making capacity (Ardisson-Korat & Rizvi, 2004; Dong et al., 2009; Papadatos et al., 2003). Whey protein-free MF-retentate is an interesting starting material for production of cheese by the FC cheese-making process.

2. Literature review

2.1. Filtration techniques

2.1.1. Basics of filtration techniques

Filtration techniques are separation methods in which components are fractionated according to size and the driving force is the pressure difference between permeate and retentate sites (Tamine, 2009). During filtration, a start material is fed to the surface of the filtration membrane and the pressure difference between the retentate and the permeate side causes permeate flow through the membrane. The type of membrane used, especially its pore size and the pressure difference determine the compositions of the final fractions (Tamine, 2009). Milk is a complex material containing many different components, and therefore filtration techniques can be used to fractionate the components (Table 1) (Brans et al., 2004). In the dairy industry, MF, UF, nanofiltration (NF) and reverse osmosis (RO) have been used to fractionate different components, as shown in Figure 1 (Brans et al., 2004).

Four membrane configurations are available for filtration systems in the dairy industry: spiral wound (SW), plate and frame, tubular and hollow fiber. The SW and tubular systems are the most widely used systems in dairy applications (de Carvalho & Maubois, 2010). SW elements consist of one or more membrane envelopes which in turn consist of two layers of membranes with a permeate spacer separating the membranes from each other. Open ends of the envelops are connected to the permeate tube. A feed channel spacer is positioned outside each membrane envelop and the whole system is rolled to form a spiral. Because of this, the hold-up volume is low and the

membrane area is large (Tamine, 2009). SW membranes are generally made with polymeric material, which is inexpensive but the limitations are limited pH range, concentration of cleaning chemicals and temperature (Tamine, 2009). In tubular systems, membrane tubes form the membrane area and the volume per square meter of membrane is higher than in the spiral type of membrane (Tamine, 2009; Walstra et al., 2006). In tubular systems the most used MF membrane type is ceramic in which the difference in pore size is narrow (de Carvalho & Maubois, 2010). Ceramic material is heat resistant which makes possible to use high-temperature heat treatments and even heat sterilization of the membranes (de Carvalho & Maubois, 2010). However, in SW polymeric systems the energy consumption and investment costs are lower than in ceramic systems (Heino, 2009; Lawrence et al., 2008), which has led to increased interest in SW systems in the dairy industry.

Table 1. The composition of milk and the sizes of its different components (data adapted from Brans et al., 2004).

Component	Concentration in milk (gkg^{-1})	Size of component (µm)
Water	871	-
Lactose	46	0.001
Fat globules	44	0.1-15
Casein	26	0.02-0.2
Whey protein	7	0.003-0.006
Minerals	7	~0.0005

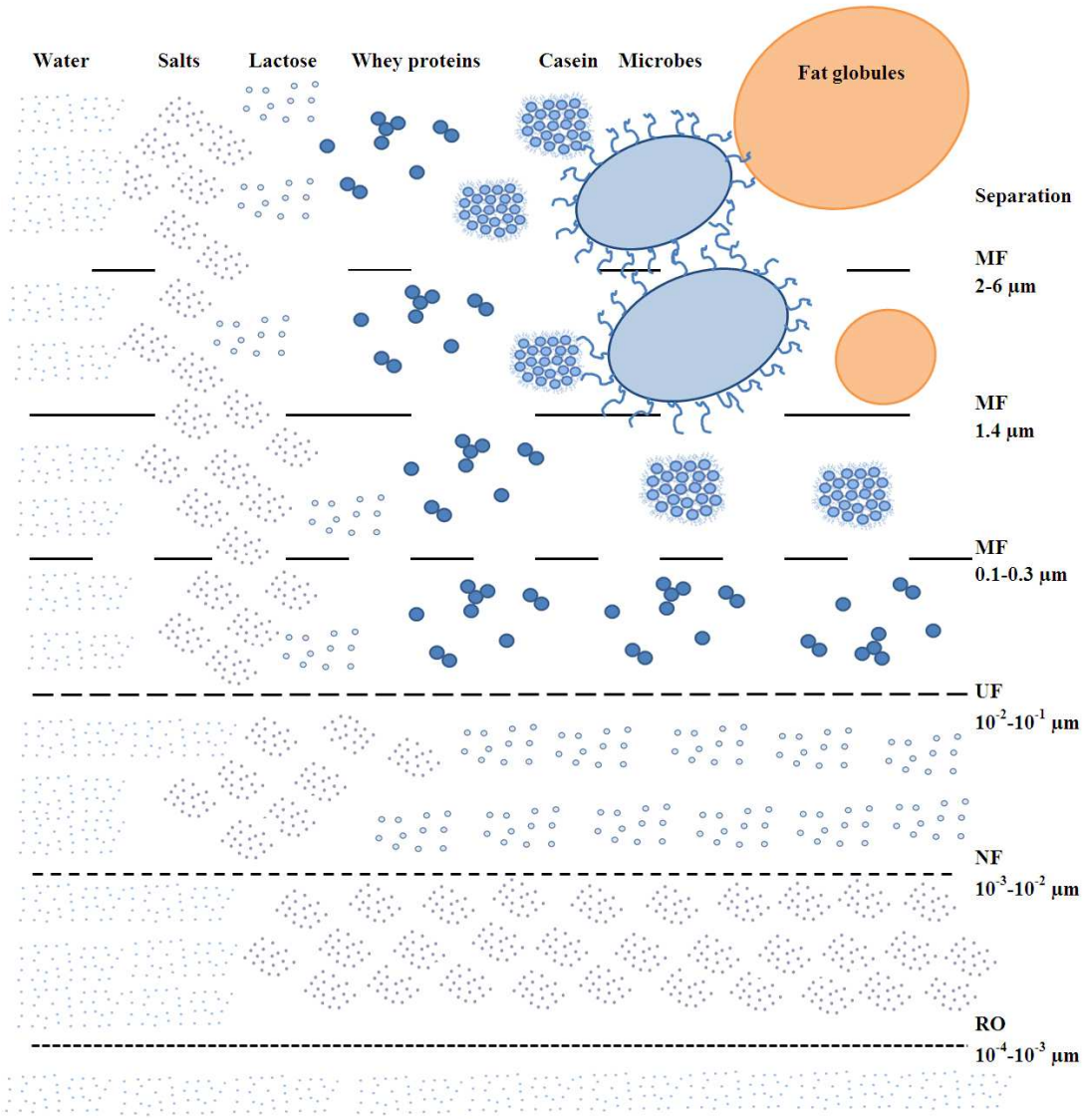

Figure 1. The components of milk can be fractionated with different filtration techniques. Fat globules of milk can be separated according to size by microfiltration (MF) with 2-6 µm pore size. The disadvantages of microbes can be avoided by concentrating skim milk by MF with a pore size of 1.4 µm. Casein and fat can be concentrated by MF with a pore size of 0.1-0.3 µm. Milk proteins and fat can be concentrated by ultrafiltration (UF), lactose by nanofiltration (NF) and salts by reverse osmosis (RO) (data adapted from Brans et al., 2004; Fox et al., 2004).

2.1.2. Microfiltration techniques

Filtration techniques in which the pore size of the membrane is 0.05-10 µm are called MF (Saboya & Maubois, 2000). Earlier, all MF membranes were ceramic and the use of these membranes was too expensive in large scale processes (Lawrence et al., 2008). However, development of filtration techniques and especially of SW membranes in MF applications made it possible to use MF in large scale (Lawrence et al., 2008).

The oldest MF application in the dairy industry is concentration or removal of microbes with 1.4 µm cutoff membranes, which has been used since the early 1980s (Madec et al., 1992; Pouliot, 2008; Saboya & Maubois, 2000). Microbes and spores of skim milk can be removed by MF from the milk, so that the heat treatment of the milk and further denaturation of whey proteins can be avoided (Fox et al., 2004). However, such bacteria-free milk has been described as too clean for cheese-making because of poor cheese flavor formation (Kelly et al., 2008).

MF with 2-6 µm pore size is used to fractionate milk fat globules (Michalski et al., 2006). The utilization of native fat globules with different sizes makes it possible to develop new products (Goudedranche et al., 2000; Michalski et al., 2003). For example, cheeses with smaller fat globules have lower lipolysis, higher melting and elastic properties and are less yellow than traditional cheeses (Michalski et al., 2003; Michalski et al., 2004).

The sizes of proteins in milk differ significantly; the main reason is that casein proteins form aggregates with each other in native milk. These aggregates, casein micelles, are significantly larger than other proteins in milk which do not form micelles (Table 2) (de

Wit, 1981). The proteins of milk can be fractionated by MF with 0.05-0.3 µm pore size (Brans et al., 2004). In this process the casein micelles are concentrated and the major whey proteins, β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), pass through the membrane (Beckman et al., 2010; Saboya & Maubois, 2000). This fractionation method is advantageous in cheese-making because the major components of cheese, fat and casein can be concentrated. The permeate is free of traditional cheese-making components, e.g. starters and organic acids, and is sterile (Gautier et al., 1994). This native whey fraction is suitable for production of highly functional whey products (Britten & Pouliot, 1996). In this study, further mention of MF will refer to filtration with 0.05-0.3 µm pore size membranes.

Table 2. The sizes of different proteins in milk (data adapted from de Wit, 1981; Vivekanand et el., 2004)

Component	Size (µm)
Casein micelles	0.03-0.3
β-Lactoglobulin	0.003-0.006
α-Lactalbumin	0.003-0.006
Bovine serum albumin	0.003-0.006
Immunoglobulin	0.005-0.01
Lactoferrin	0.009

Previously, all scientific studies of separation of casein micelles and whey proteins have been made using ceramic membranes (Brandsma & Rizvi, 1999; Britten & Pouliot, 1996; Caron et al., 1997; Garem et al., 2000; Larsson et al., 2006; Nelson & Barbano, 2005a; Neocleous et al., 2002ab; Samuelsson et al., 1997). However, development of SW membranes has made it possible to separate proteins more economically (Heino, 2009; Lawrence et al., 2008), and nowadays there are more studies in which whey

proteins and casein micelles have been separated with SW membranes (Govindasamy-Lucey et al., 2007; Heino, 2009; Heino et al., 2010; Hurt & Barbano, 2010; Lawrence et al., 2008)

2.1.3. Ultrafiltration, nanofiltration and reverse osmosis techniques

UF (pore size 10^{-2}-10^{-1} μm) can be used to concentrate proteins and fat while lactose and salts pass through the membrane. For over 30 years, UF has been used (Maubois & Mocquot, 1974) in soft cheese production and in the protein standardization of cheese milk (Govindasamy-Lucey et al., 2011; Guinee et al., 2006). It is possible to increase cheese yield using UF, with the result that whey protein recovery to cheese increases (Brown & Ernstrom, 1982; Govindasamy-Lucey et al., 2011; Jameson & Lelievre, 1996; Maubois & Mocguot, 1974). In protein standardization processes, the protein content of cheese milk is increased with UF or MF which increases the cheese yield from the cheese vat and increases the capacity of the cheese-making process (Govindasamy-Lucey et al., 2005; Neocleous et al., 2002a). Whey proteins affect negatively cheese texture and ripening (Christensen et al., 1991; Harper et al., 1989; Lelievre et al., 1990; Sutherland & Jameson, 1981), and for this reason the FC process with UF has not been used in the production of semi-hard and hard cheeses (Horton, 1997; Vivekanand et al., 2004).

RO has also been used in the concentration of cheese milk (Bynum & Barbano, 1985). However, the disadvantage of RO is its high driving pressure, which homogenizes the milk fat and decreases fat recovery in cheese-making (Bynum & Barbano, 1985). RO also increases the lactose content of retentate, which may cause off-fermentation and d-Ca-lactate crystallization on the surface of cheese (Fox et al., 2004). For these reasons

RO has not commonly been used in cheese-making. UF, NF and RO have been used in whey processes in order to achieve more valuable products and economical processes (Marshall, 1997; Robin et al., 1993).

2.1.4. Diafiltration techniques

Retentate dilution and diafiltration (DF) can be used in order to improve fractionation of components and to remove components from the retentate (Tamine, 2009). DF can be used in combination with any other filtration techniques except RO. The solution used for dilution of the retentate is called diawater and is commonly water or any other solution which does not include the components which will be removed from the retentate. DF has been used for example to remove whey proteins from MF-retentate (Larsson et al., 2006; Nelson & Barbano, 2005b), to reduce the lactose or mineral content of retentate (McMahon et al., 1997; Spangler et al., 1991) and to reduce the buffering capacity of retentate (St-Gelais et al.,1992a).

2.2. Evaporation techniques

Evaporation is commonly used to concentrate milk before drying or transportation (Farkey, 2008). Evaporation is a unit operation in which water is removed (Tamine, 2009) at reduced pressure, primarily in order to allow boiling at lower temperature and thus prevent damage due to heating (Walstra et al., 2006). Evaporation is considered to be one of the most energy-intensive processes in the dairy industry (Farkey, 2008). It is well known that filtration techniques are more economical concentration methods than evaporation (Valentas et al., 1997). However, it is not possible to reach high total solids with filtration techniques and therefore high total solids can be reached only by

evaporation (Figure 2). Highly viscous products can be evaporated by the scraped surface evaporation method in which a rotating blade wipes the heated surface in order to promote better heat transfer. When the final total solids increases further the only possible concentration method is drying (Figure 2).

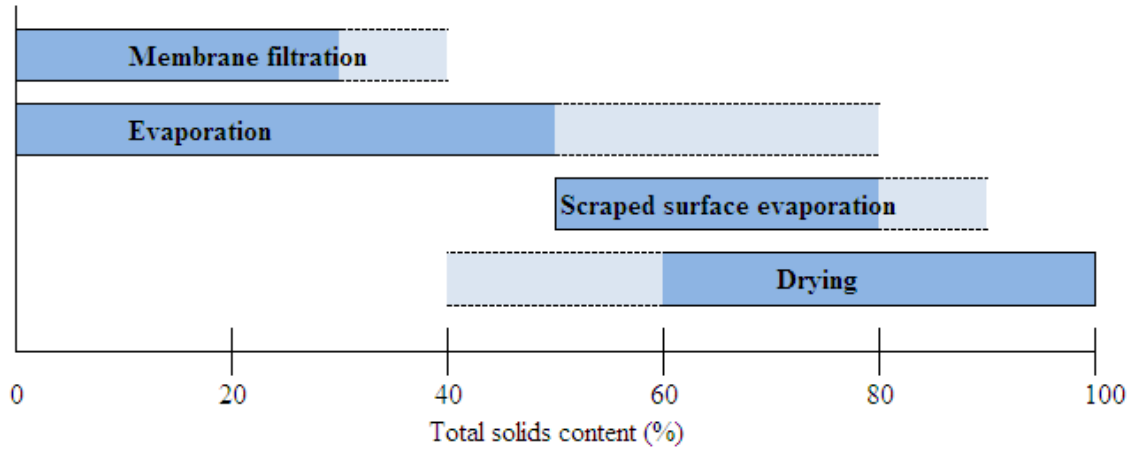

Figure 2. Suitable concentration methods for processes reaching different total solids contents (modified from Morrison & Hartel 2006).

2.3. Cheese-making with concentrated milk

2.3.1. Standardization of components of retentate

Basics of cheese-making processes

Traditionally, cheeses are made with a process in which milk is acidified and coagulated with a coagulant. Coagulated milk is cut to small pieces and the cheese curd is formed when the whey is separated from the casein and fat fraction. The curd is cooked for a specified time depending on the cheese variety. The whey is drained and the curd is moulded, pressed, brine salted and ripened. The final chemical content of the cheese depends on all the described process steps (Fox & McSweeney, 1998). Basically, the

higher the moisture content of cheese, the higher is its content of whey components, for example lactose and whey proteins (Fox et al., 2000). In the FC process, no whey draining is used and the retentate must be standardized in terms of whey protein, lactose, calcium, salt, fat and total solids before cheese-making (Ernstrom et al., 1980; Jameson & Sutherland, 1994). The process steps of traditional cheese-making and cheese-making from FC milk are shown in Figure 3.

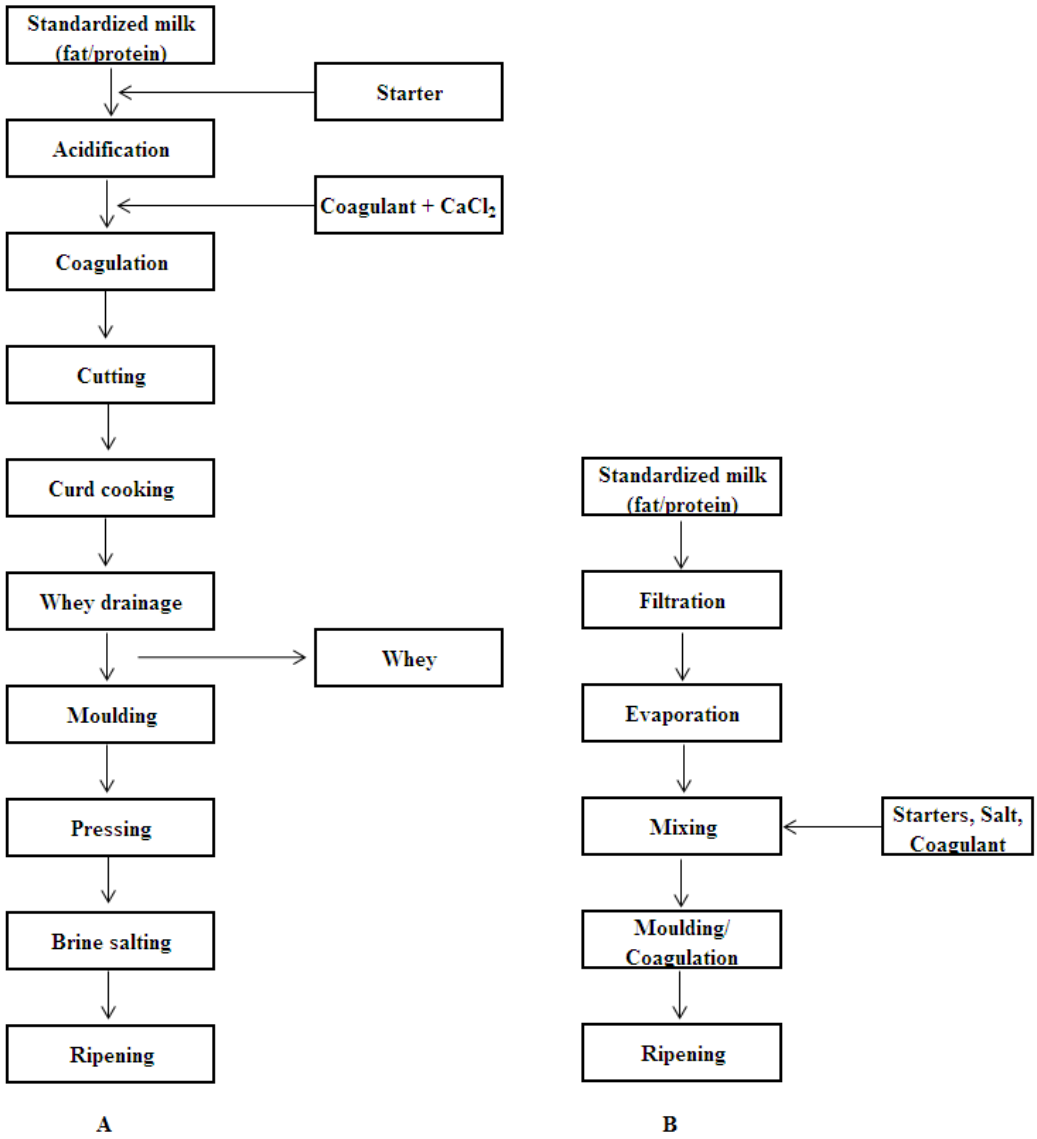

Figure 3. The process of traditional cheese-making (A) is more complex and includes more process steps than cheese-making from full concentrated milk (B).

Whey protein removing

In traditional cheese-making, casein micelles are hydrolyzed by chymosin and casein micelles lose their solubility thus creating a gel structure. The soluble proteins or whey proteins are removed with whey from the gel, which is then cut into small pieces (Fox & McSweeney, 1998). The main whey proteins, β-LG and α-LA, can be removed from the MF-retentate by DF without any effect on the concentrations of other components when UF-permeate is used as diawater (Heino, 2009; Larsson et al., 2005; Maubois, 2002; Nelson & Barbano, 2005a). However, the MF-membrane retains lactoferrin, bovine serum albumin (BSA) and immunoglobulins (Jost et al., 1999; Outinen et al., 2010). Caseinomacropeptides are also retained in MF-retentate (Outinen, 2010), because they are released from casein micelles by the coagulant (Fox & McSweeney, 1998). Therefore during cheese-making from FC retentate, the coagulation step is performed after concentration to the final total solids content.

Effect of lactose on cheese

In traditional cheese-making, the seasonal changes of lactose content are high and lactose content is standardized by whey removal and water addition during cooking (Shakeel-Ur-Rehman et al., 2004; Upreti & Metzger, 2006). Too high lactose content causes increased lactic acid formation and too low pH (Shakeel-Ur-Rehman et al., 2004). In cheese, lactose residues can cause loss of solubility of the d-Ca-lactate, which crystallizes on the surface of cheese (Fox et al., 2000). Too low lactose content causes reduced lactic acid formation (Heino et al., 2008) and affects cheese flavor. In traditional cheese-making, about 96% of the lactose in milk is removed with whey as lactose or lactic acid (Fox et al., 2000). Fermented lactose contents in Dutch cheeses are 2.9% of total solids (Fox et al., 2000). If the cheese is made from FC milk retentate, no

curd formation or whey draining is made and the lactose content must be reduced before cheese-making to avoid d-Ca-lactate crystallization (Sutherland & Jameson, 1981). The lactose content of retentate can be reduced with a DF step (McMahon et al., 1997).

Effect of calcium on final cheese product

In milk, 68% of calcium and 47% of phosphates are present as colloidal ions in casein micelles and the remainder is in soluble form (Lucey & Fox, 1993). During pH decrease, calcium and phosphate become soluble, which increases the buffering capacity of milk (Fox & McSweeney, 1998). Milk concentration with UF or MF increases the calcium content and buffering capacity of the retentate and therefore the pH reduction is slower than in traditional cheese-making (McMahon et al., 1997; Mistry & Kosikowski, 1986). Because of its higher buffering capacity, cheese made from concentrated milk requires more starter or acid to reach the optimal pH (Johnson & Lucey, 2006). High buffering capacity has been reported to decrease the proteolysis of cheese by reducing the lysis of starter (Mistry & Kosikowski, 1986; St-Gelais et al., 1995). The calcium and phosphate contents of cheese also affect its textural and functional properties (Joshi et al., 2003; Sheehan, & Guinee, 2004). The final contents of calcium and phosphate in traditional cheeses are consequences of processing and especially the pH of cheeses, as well as the soluble calcium concentration in cheese milk during coagulation and cooking (Lucey & Fox, 1993; Johnson & Lucey, 2006). Calcium content affects the textural properties of the final product; the harder the cheese, the higher is its content of calcium (McMahon et al., 2005). Thus different varieties of cheese have different calcium-protein ratios as shown in Table 3 (Lucey & Fox, 1993). Cheese milk concentration increases the content of casein micelles and the final calcium content (Brandsma & Rizvi, 1999; Karlsson et al., 2005; Schreier et al.,

2010; St-Gelais et al., 1992a), which increases whey draining during cheese ripening (Delbeke, 1987; Pastorino et al., 2003).

Table 3. Calcium-protein ratio in different cheese varieties (data adapted from Lucey & Fox, 1993).

Cheese variety	Ca:Protein (mg g^{-1})
Cottage	5.4
Camembert	18.2
Edam	29.4
Cheddar	31.5
Gouda	32.2
Emmental	33.1

Standardization of calcium content is essential during cheese-making from concentrated or FC milk. The calcium content is decreased by DF when water or salt solution is used as a diawater (Spangler et al., 1991; McMahon et al., 1997). During DF, soluble minerals pass through the membrane and decrease the buffering capacity of milk (St-Gelais et al., 1992b). However, quite a large amount of calcium ions are bound to casein micelles and thus DF may not decrease the calcium content sufficiently. The decrease of calcium content can be intensified by acidification before filtration, which increases the solubility of the calcium and more calcium pass through the membrane (Brandsma & Rizvi, 1999, Eckiner & Zottola, 1992; Ernstrom et al., 1980). However, in previous studies it has been observed that milk acidification before filtration increased membrane fouling and reduced the flux (Brandsma & Rizvi, 1999; Eckiner & Zottola, 1992; Ernstrom et al., 1980; St-Gelais et al., 1992a). It is known that membrane fouling begins when whey proteins form a layer on the membrane surface and free calcium ions are able to bind to the whey protein layer in addition to the casein micelles (Vetier et al.,

1988). Acidification and microfiltration of whey protein-free retentate has not been studied previously.

2.3.2. Processability of full concentrated cheese retentate

Viscosity of concentrated milk

During cheese-making from FC milk, the content of milk is standardized and it is concentrated to the final total solids content of cheese. It is well known that increased total solids decreases the distance between casein micelles and increases the viscosity of retentate (de Kruif, 1997). During evaporation of milk, the viscosity of the concentrate increases exponentially as a function of total solids and at the same time evaporation causes structural changes in milk (Velez-Ruiz & Barbosa-Canovas, 1998; Walstra et al., 2006). However, the denaturation of whey proteins, salt content and pH affect the rheological properties of the concentrate (Anema et al., 2004; Karlsson et al., 2005). Anema et al. (2004) reported that acidification and heat treatment of milk increased its viscosity and the size of the casein micelles. It has been shown that whey proteins bind to casein micelles more easily at lower pH values (Corredig & Dalgleish, 1996). Wade et al. (1996) also observed increased size of casein micelles during acidification of milk, although they used acid to decrease the pH, which may have affected the stability of casein micelles. When glucono-δ-lactone (GDL) was used as an acidifying agent, a slight decrease in the average particle size was observed until the pH reached 5.55 (Banon & Hardy, 1992). The chemical composition of milk, heat treatment and acidification methods greatly affect the rheological properties of the final concentrated product. The effect of pH on casein micelles has been studied in the absence of other components of milk and it has been observed that casein micelles have their most

compact structure at pH 5.5 (Liu, & Guo, 2008). The viscosity of FC pre-cheese retentate must be reduced or controlled so that mixing and moulding steps can be carried out.

Coagulation properties

In concentrated milk, the components are physically close to each other and casein aggregation is rapid (Thomann et al., 2008). However, it has been reported that native whey proteins inhibited the activity of coagulant (Lelievre et al., 1990). Inhibition by whey proteins can be avoided using MF to remove these proteins. It has been found that MF concentrate coagulates rapidly because the number of structure-forming particles is high, and therefore the amount of coagulant can be reduced (Dong et al., 2009).

In traditional cheese-making, the pasteurization of milk has a positive effect on its microbiological quality by destroying pathogenic bacteria and reducing different off-fermentations during cheese ripening. However, high pasteurization temperatures, above 75^{o}C, cause denaturation of whey proteins, and when whey proteins bind to the surface of casein micelles (Jang & Swaisgood, 1990) the release of CMP decreases, coagulation time increases and gelling properties decreases (Singh & Waungana, 2001; Vasbinder et al., 2003). Schreiber and Hinrichs (2000) studied coagulation properties of MF milk. They found that when the whey proteins were removed, the retentate could be pasteurized at 110^{o}C without detrimental effects on the coagulation of milk. Microbiological quality of MF retentate can be improved with high pasteurization, however too clean starting material decreases the flavor intensity of the final cheese product and increases the requirements for starter.

2.3.3. Lactose fermentation with lactic acid bacteria

The fermentation of lactose has a great impact on the cheese process and on the texture and flavor of the final product. The main LAB, for example *Lactococcus lactis* sp. *cremoris* or *lactis*, use the glycolytic pathway, with L-lactic acid as a main product (1). However, there are also other LAB for example *Leuconostoc mesenteroides,* which use the phosphoketolase pathway for lactose fermentation and produce D-lactic acid, acetic acid or ethanol and CO_2 (2). Ethanol and acetate have positive impacts on cheese flavor and CO_2 is responsible for eye formation in cheese. In Dutch-type of cheese, mesophilic *L. lactis* sp. *lactis, L.lactis* sp. *cremoris* and *Leuc. mesenteroides* are used as starter bacteria (Fox & McSweeney, 1998).

$$\text{Lactose} \xrightarrow{\text{Glycolytic pathway}} 4\text{L - lactic acid} + 4\text{ATP} \qquad (1)$$

$$\text{Lactose} \xrightarrow{\text{Phosphoketolase pathway}} 2\text{D - lactic acid} + 2\text{acetic acid/ethanol} + CO_2 \qquad (2)$$

2.3.4. Cheese ripening

Basics of cheese proteolysis

Cheese ripening consists of a complex biochemical reaction series in which fermentation of lactose and citrate and breakdown of fat and proteins are the main routes to the production of flavor components (Marilley & Casey, 2004; McSweeney, 2004). It has been proposed that protein breakdown to peptides and free amino acids (FAA) and conversion of FAA to flavor components are the main pathways to flavor formation (Yvon et al., 1997). Primary proteolysis begins when the indigenous enzyme plasmin (PL) and added chymosin hydrolyze proteins to smaller peptides (Fox, 1989;

Visser, 1993) (Table 4). During secondary proteolysis, peptides are further hydrolyzed to smaller peptides and FAA by the action of enzymes of starter and non-starter bacteria (Figure 4) (McSweeney, 2007).

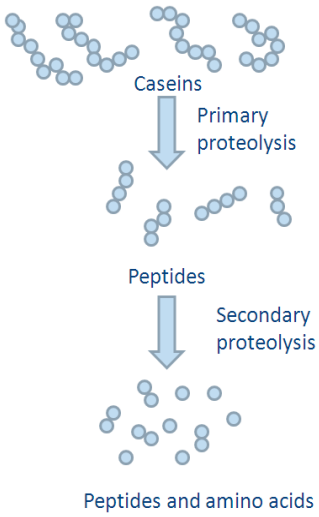

Figure 4. The proteolysis of cheese begins when the indigenous enzyme of milk, plasmin, and added chymosin hydrolyze casein to peptides. Peptides are further hydrolyzed to smaller peptides and free amino acids by the peptidases of lactic acid bacteria (data adapted Marilley & Casey, 2004).

Table 4. Casein proteins are hydrolyzed to peptides by chymosin and plasmin (data adapted from Fox & McSweeney, 1998; Kelly & McSweeney, 2003).

Substrate	Hydrolytic agent	Product
κ-casein	chymosin	para-κ-casein macropeptide
β-casein	plasmin (+chymosin)	$\gamma_{(1-3)}$-casein proteose peptones
α_{S1}-casein	chymosin (+plasmin)	λ-caseins proteose peptones
α_{S2}-casein	plasmin	several unidentified peptides

Effect of chymosin on cheese proteolysis

Most cheeses are produced using an enzymatic coagulation in which added enzyme hydrolyzes the Phe_{105}-Met_{106} bond of κ-casein. When 85% of milk κ-casein has been hydrolyzed, the stability of casein micelles decreases and coagulation of milk will begin if the temperature is above 20°C (Fox & McSweeney, 1998). A commonly used coagulant, chymosin, also hydrolyzes α_{s1}- and to some extent β-casein during ripening. It has been shown that off-flavor and the bitterness of cheese are due to hydrophobic peptides which are mainly released from α_{s1}-caseins (Lee et al., 1996). An increased amount of chymosin increases the bitterness of cheese (Fallico et al., 2005; Spangler et al., 1991) but also affects its structural properties. Increased chymosin level reduces cheese hardness and increases the meltability of cheese (Dave et al., 2003). In traditional cheese-making, most of the chymosin activity is lost in the whey and only 0-15% remains in cheese (Guinee & Wilkinson, 1992). During cheese-making from FC milk retentate, no whey removal is made after the addition of chymosin and the activity of chymosin may be higher than in traditionally made cheese during ripening (Voutsinas et al., 1995). However, chymosin is not heat stable and it is inactivated at temperature above 50°C. The process of deactivating the chymosin enzyme by heat treatment of cheeses in packages at 55-60°C has been patented (Altamirano et al., 1999). This kind of treatment can be used in FC processes to avoid bitterness (Altamirano et al., 1999). The bitterness of cheese can also be reduced by increasing the secondary proteolysis, for example by adding peptidases extracted from LAB (Lee et al., 1996) or by reducing the chymosin level.

Effect of plasmin on cheese proteolysis

The indigenous enzyme of milk, plasmin (PL) (fibrinolysin, fibrinase, EC 3.4.21.7), is a protease which is important in the primary proteolysis of cheese. However, too high PL activity may cause some disadvantages because too early proteolysis may decrease the cheese yield (Mara et al., 1998). PL with its inactive coenzyme plasminogen (PG), originates from bovine blood. There is also a complex network with PL activators, PG inhibitors and inhibitor activators in milk (Fox & McSweeney, 1998) (Figure 5).

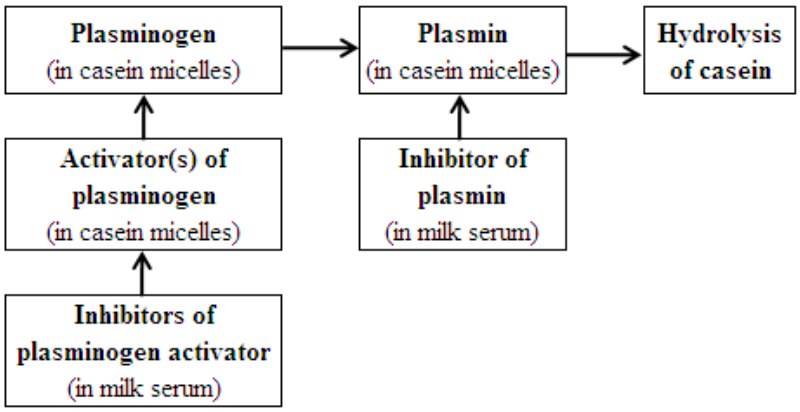

Figure 5. In milk, there are complex interactions between plasmin activators and inhibitors which affect plasmin activity and the hydrolysis of casein (data adapted from Fox & McSweeney, 1998)

PL hydrolyzes specifically a peptide bond between lysine and arginine, particularly in β-casein and α_{s2}-casein, and it also has a slight activity against α_{s1}-casein (Aslam & Hurley, 1997; Crudden et al., 2005; McSweeney et al., 1993; Trujillo et al., 1997). Native PL and PG are stable at sterilization temperatures (Rollema & Poll, 1986). However, ultrahigh-temperature sterilization (UHT) completely destroys PL activity in milk (Korycka-Dahl et al., 1983) Thermal inactivation of PL is also significant at 90^{o}C in milk (Benfeldt et al., 1997; Prado et al., 2007; Saint Denis et al., 2001). The free thiol-disulfide group of denaturated β-LG binds with the thiol-rich structures of PL and

PG (Rollema & Poll, 1986) and thus inactivates PL and PG at high temperatures (Saint Denis et al., 2001).

Cheese-making from UF-retentate has shown reduced proteolysis during ripening (Christensen et al., 1991). It has been proposed that the increased concentration of whey proteins decreases the activity of PL in cheese made from concentrated milk (Bastian et al., 1991). However, Benfeldt (2006) found that the concentration factor (CF) of UF-retentate did not affect the PL activity and assumed that PL activity was inactivated during the filtration and was dependent on filtration time, temperature and feed line pressure in the equipment. Cheese-making from MF retentate has also indicated decreased proteolysis of cheese in low CF (Neocleous et al., 2002b). However, cheese made from whey protein-free MF-retentate showed increased proteolysis (Nelson & Barbano, 2005b) and removal of whey proteins has been observed to result in an increase in concentration of small peptides, which may be the result of PL activation (Nelson & Barbano, 2005a).

Secondary proteolysis

Starter and non-starter LAB greatly influence cheese proteolysis, as shown in Figure 6. Proteases in the starter cell wall are able to hydrolyze caseins to smaller peptides which the cell can take up with the aid of transporters (Fox et al., 2004). Small peptides and FAA are converted to flavor components inside the cell. However, lysis of the LAB also has important effects on cheese flavor (Hannon et al., 2003; Lepeuple et al., 1998). During the starter lysis, peptidases are released to the cheese matrix and free peptides are hydrolyzed to smaller peptides and FAA (McSweeney, 2007). It is well known that increased starter lysis and released peptidases reduce the bitterness of cheese (Chapot-

Chartier et al., 1994; Lee et al., 1996). Increased primary proteolysis does not involve the formation of FAA and therefore the secondary proteolysis greatly impacts proteolysis in cheese (Milesi et al., 2008).

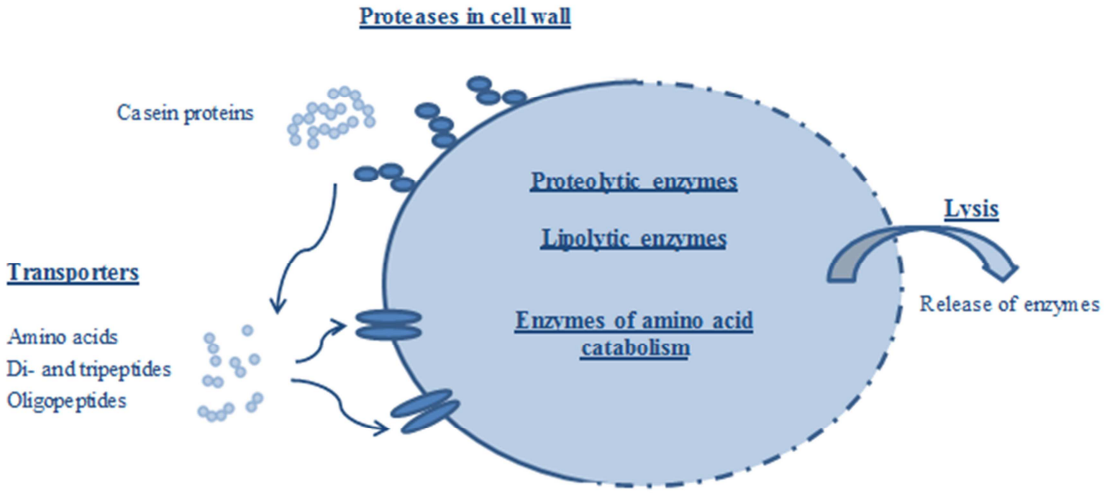

Figure 6. Proteases in the starter cell wall hydrolyze casein proteins to peptides. The cell takes up small peptides and free amino acids and uses them in catabolic reactions in which different flavor components are released. Starter lysis also has a positive effect on the proteolysis (modified from Fox et al., 2004; McSweeney, 2007).

Starter lysis can be estimated by measuring the viable cell count from cheese (Crow et al., 1995a). However, this method only demonstrates decreases in viability, not the release of intracellular enzymes (Crow et al., 1995a). A more efficient method is the detection of intracellular enzymes, such as lactate dehydrogenase (LDH), glucose-6-phosphatase dehydrogenase (G6PDH), X-prolyl-dipeptidase (Pep-X) and post-proline dipeptidyl amino peptidase (PPDA) in cheese matrix (Hannon et al., 2006; Lepeuple et al., 1998, Wilkinson et al., 1994). During starter lysis, release of enzymes has been detected with activity measurement and by immunoblotting. Autolysis marker enzymes have different stabilities; it has been shown that G6PDH and PPDA lose their activity in

cheese slurry at 4^{o}C within 24 h, whereas LDH activity remains stable for 500 h (Wilkkinson et al., 1994).

During cheese ripening, lysis of the starter may be delayed when cheeses are made from concentrated milk (Hannon et al., 2006; Mistry & Kosikowski, 1986; Saboya et al, 2001; St-Gelais et al., 1995). Two possible reasons have been presented for reduced lysis of the starter. One reason is too high nutrition content and the other is too high buffering capacity (Mistry & Kosikowski, 1986; Riepe et al., 1997; St-Gelais et al., 1995). Both aspects affect starter growth and improve the viability of bacteria. High nutrient content and buffering capacity can be reduced with a DF step in which the contents of lactose and minerals are decreased (McMahon et al., 1997; St-Gelais et al., 1992b). There are also other methods to increase peptidase activity in cheese. Starter strains have very different lysis behavior and lysis can be improved by selecting suitable starter strains (Hannon et al., 2006). It is also possible to improve peptidase activity, reduce cheese bitterness and accelerate cheese ripening by adding heat- or freeze-shocked or permeabilized starter strains (Abboudi et al., 1991; Bech, 1993). One possibility to increase secondary proteolysis is the addition of peptidase enzymes. Enzyme additions have not been adapted in traditional cheese-making because of loss of activity during curd washing and brine salting (Wilkinson & Kilcawley, 2005). However, these problems are not relevant in cheeses made from FC retentate, when no whey removal or brine salting is made after enzyme addition and the enzyme activity remains in cheese.

3. Aims of this study

There are no reports of the production of hard or semi hard cheeses from whey protein-free milk with FC processes. The primary aim of this study was to develop a process for the production of semi-hard cheeses from FC milk. In pursuit of this aim, the following activities were undertaken: 1) standardize concentrated milk, 2) determine how standardization affects the composition of concentrated milk and 3) investigate how biochemical and microbiological reactions, fermentation and proteolysis, were altered when cheeses were made from concentrated milk.

In study I, the focus was to determine, how MF of milk and whey protein removal affect the PL and PG-derived activities in retentate. It was also investigated how high heat-treatment affects PL and PG-derived activities in whey protein-free retentate.

In study II, the aim was to determine how the calcium content of the pre-cheese retentate can be standardized with acidification and filtration so that the calcium-protein ratio is suitable for different cheese varieties.

In study III, the aim was to determine the effect of pH on the viscosity of concentrated whey protein-free retentate. Moreover, the purpose was to show how the viscosity of the retentate can be reduced so that FC retentate is easier to handle in cheese-making. It had previously been reported that the acidification of milk before filtration reduced filtration flux (Brandsma & Rizvi, 1999; Eckiner & Zottola, 1992; Ernstrom et al., 1980; St-Gelais et al., 1992a) and therefore the effect of acidification on filtration flux was also studied.

Delayed lysis of LAB and reduced secondary proteolysis is known to occur in cheeses made from concentrated milk. This lag of peptidase activity can be compensated by addition of enzymes. In study IV, the aim was to determine how added peptidases affect the ripening of FC cheese.

In study V, the aim was to determine how added $CaCl_2$ solution affected starter viability in cheese made with the FC process.

4. Materials and Methods

4.1. Development of full concentration process

The FC process was developed step by step during the studies (Figure 7A). In study I, the effect of whey protein removal and lactose standardization on PL activity was studied. Calcium standardization was studied in study II and viscosity reduction in study III. The FC cheese-making process was used to produce cheeses in studies IV and V and the FC process used is shown in Figure 7B.

4.1. Pretreatment of milk

In studies I, II and III the milk was obtained from a local dairy (Valio Ltd, Riihimäki, Finland). Milk was pasteurized at 72^{o}C for 15 s and the fat-protein ratio was standardized to 0.7:1. The pasteurized milk was cooled to filtration temperature (50^{o}C). In studies IV and V, the skim milk was obtained from another local dairy (Valio Ltd, Lapinlahti, Finland). Milk was pasteurized at 72^{o}C for 15 s, cooled to filtration temperature (15^{o}C) and used as a feed in further filtrations.

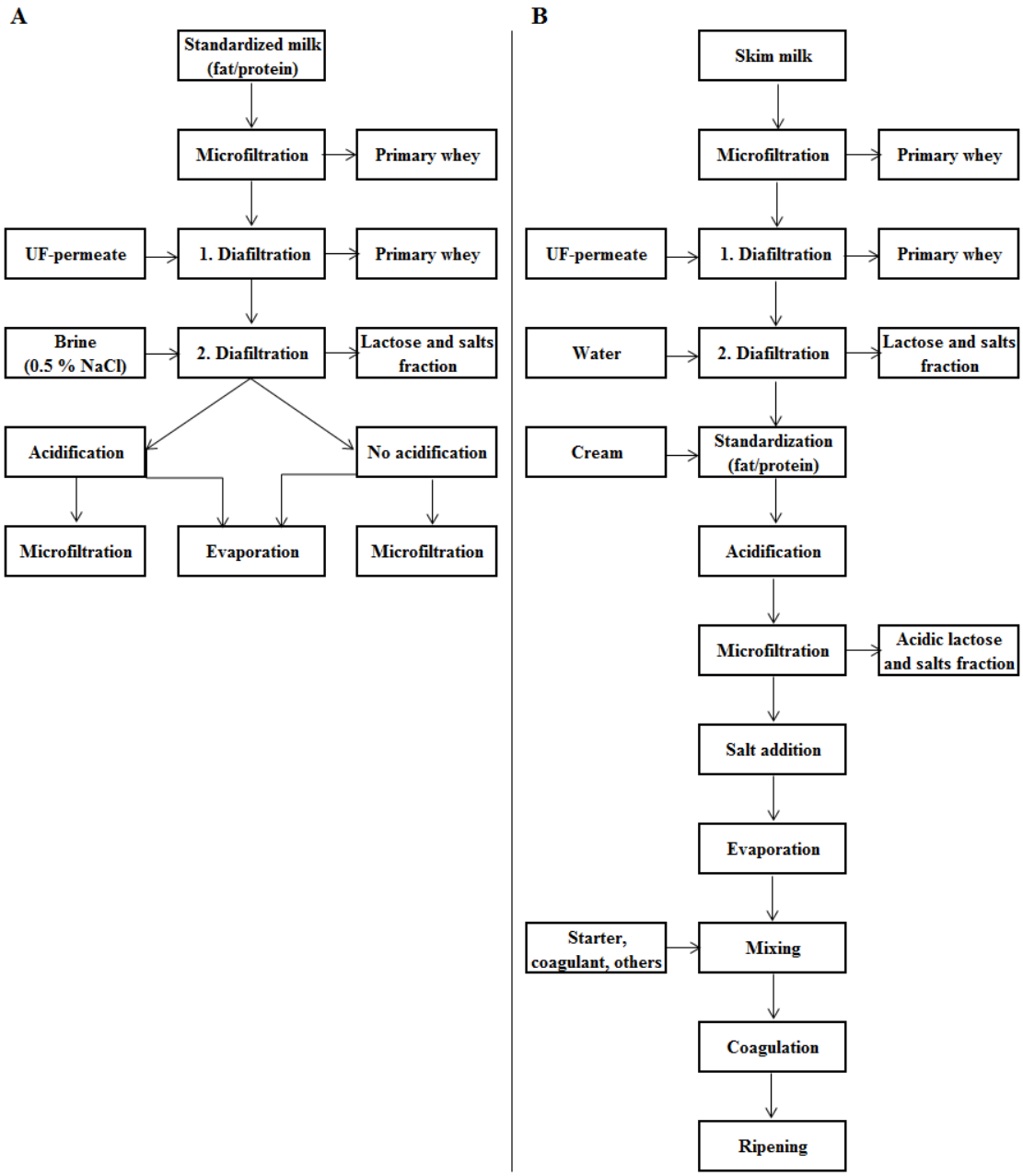

Figure 7. The FC process was developed by investigating the whey protein removal, calcium standardization and viscosity behavior of concentrate produced according to process A (studies I, II, III). The final FC process (B) was used in studies IV and V.

4.2. Membrane filtrations

4.2.1. Whey protein removal and lactose standardization (I-V)

Pretreated milk was used as a feed in MF and concentrated by recirculation of the milk through SW membranes with an 800 kDa cutoff membrane (FR-3 A-6338-PHT, Synder Filtration, Vacaville, USA) in a Multipurpose Pilot plant (APV, Silkeborg, Denmark). The filtration was performed at 50^{o}C in studies I-III and at 15^{o}C in studies IV and V. The feed was concentrated to CF 4 and after concentration the milk was diafiltrated. In studies I-III, the whey proteins were removed with the first DF step in which 150 kg of MF-retentate was diafiltrated with 1000 kg of UF-permeate. The lactose content was standardized with a second DF step in which 170 kg of brine (0.5% w/v, NaCl) was used as diawater. In studies IV and V, whey proteins were removed from retentate (275 kg) by DF in which 1830 kg of UF-permeate was used as diawater. The lactose standardization was performed with a DF step in which 330 kg of water was used as diawater. Final retentates were pasteurized at 95^{o}C for 15 s in all studies. Fat-protein ratio was standardized to 0.95:1 with high pasteurized (at 95^{o}C for 15 s) cream obtained from a local dairy (Valio Ltd, Lapinlahti, Finland) in studies IV and V.

4.2.2. Acidification of retentate (II-V)

In studies II-IV, the retentates were acidified with mesophilic starter cultures (DVS-R608, Chr-Hansen, Hoersholm, Denmark). Retentates were inoculated with 0.1% (w/w) of starter cultures and incubated at 33^{o}C until the target pH was reached. The acidification was stopped by heating the retentate to 50^{o}C. The target pH was 5.75 in study II, 6.0 and 5.75 in study III and 5.8 in study IV. The effect of acidification on

proteolysis of retentate was evaluated with a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method in study III. Retentates were used as a feed in second filtration in all studies; however in study III it was also used as feed in evaporation.

4.2.3. Filtration of acidified retentate (II-V)

In studies II and III the acidified and non-acidified retentates were further concentrated using ceramic MF membranes with molecular cut-off 0.1 µm (Membralox P19-40, Pall, Bazet, France). Filtration was performed at 50^{o}C, uniform transmembrane pressure (UTP) was 70 kPa in Tetra Alcross MFS-1 (Tetra Pak, Copenhagen, Denmark). Feeds were concentrated to CF 1.8. Concentrated retentates of non-acidified feeds were acidified as described in the previous section.

In study IV, the acidified retentates were concentrated with SW membranes at 50^{o}C according to the first filtration step. Retentates were concentrated to CF 2.2. Concentrated retentates were salted with solid salt so that the salt content of total solids was 2.9%. Salted concentrates were used as a feed in evaporation.

4.3. Evaporation of whey protein-free milk retentate (III-V)

In study III, non-acidified and acidified retentates were evaporated with a Stephan vat (UMC 5 electronic, Hameln, Germany). The vacuum pressure was maintained at 60 kPa, evaporation was at 70^{o}C and the retentates were mixed during evaporation.

In studies IV and V, prefiltrated retentates were evaporated in a Convap test unit (Alfa Laval, Newburyport, USA). Evaporations were performed under 0.75 bar vacuum at 70^{o}C. Retentates were evaporated to 480 g kg^{-1} total solids content.

4.4. Cheese-making from full concentrated retentate (IV, V)

Evaporated cheese retentates were inoculated with 0.1 g kg^{-1} DVS-CHN-19 starter cultures (Chr.-Hansen) (contains *L. lactis* subsp *lactis*, *L. lactis* subsp *cremoris*, *Leuc. mesenteroides*, *L. lactis* subsp *lactis* var. *diacetylactis*) and with 0.05 g kg^{-1} chymosin (Chy-Max Extra, Chr.-Hansen) at 45^{o}C. Some of the cheese concentrates were inoculated with 0.5 g kg^{-1} of permeabilized peptidase-active *Lactococcus lactis* strain (peptidases) (FlavoCard, Danisco, Copenhagen, Denmark). Cheese concentrates were mixed well after additions and were coagulated for 1 h. After the coagulum was formed, cheeses were pressed for 4 h at 22^{o}C. Cheeses were vacuum-packed and ripened at 12^{o}C for 8 weeks and ripening was monitored. The basic compositions of FC cheeses are shown in Table 5.

In study V, the effect of $CaCl_2$ addition on starter viability was studied by increasing the calcium-protein ratio from 20 mg g^{-1} to 31 mg g^{-1} in some of the cheeses. The calcium-protein ratio was increased by adding 340 g kg^{-1} $CaCl_2$ solution with starter and chymosin addition to some of the cheeses. Cheeses were ripened according to study IV.

Table 5. Chemical contents of cheeses made from full concentrated milk on day one (n=3).

Component	Concentration (g kg^{-1})
Protein	210.0 ± 4.0
Fat	230.0 ± 20.0
Total solids	480.0 ± 20.0
Lactose	5.9 ± 0.6
Lactic acid	1.5 ± 0.1

4.5. Methods of analysis

4.5.1. Evaluation of filtration

The filtrations were evaluated by calculating CF according to equation (3).

$$CF = V_F / V_R , \quad (3)$$

where V_F is the volume of feed, V_R the volume of retentate. The effects of filtrations on different components were evaluated with equations (4) and (5) during the studies.

$$\text{Retention ratio} = C_R / C_F , \quad (4)$$

$$\text{Permeation ratio} = C_P / C_R , \quad (5)$$

where C_R is the content of component in retentate, C_F the content of component in feed and C_P the content of component in permeate.

4.5.2. Chemical and microbiological analyses

Chemical and microbiological analyses of samples are presented in Table 6. The analyses of the specific whey proteins β-LG and α-LA were performed using reversed-phase high-performance liquid chromatographic (RP-HPLC). A more detailed description is presented in study I. Calcium content was analyzed using an inductively coupled plasma-mass spectrometer (ICP-MS Elan 6100; Perkin Elmer, Concord, Canada) as described in study II.

Table 6. Chemical and microbiological analyses of samples.

Analysis of	Reference
Total solids (TS)	IDF(1987)
Total nitrogen (TN)	IDF(2001a, 2001b)
Total protein (TP)	IDF (2001b)
Fat	IDF (1996, 2008)
Lactose	IDF (2002) with modification
Chloride	IDF (2006)
Non-protein nitrogen (NPN)	IDF (2001c)
Casein (CN)	IDF (2004)
Native whey protein	[TN-(CN-NPN)]x 6.38
Lactic acid	IDF (2005)
Total lactic acid bacteria count	IDF (2010)
Acetic acid	de Jong, & Badings (1990) with modification
Titrable free amino acid (FAA)	Moisio, & Heikonen (1996) with modification

4.5.3. Protein profile analysis

In study III, the protein profiles were analyzed with SDS-PAGE. The samples were diluted in water to a final protein concentration of 17 g kg^{-1}. The samples were diluted 1/2 in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 1/6 in 1% (v/v)

mercaptoethanol and 1/3 in 10% (v/v) SDS solution. The solutions were heated in boiling water for 5 min, 0.007% bromphenol blue was added and the solutions were mixed and centrifuged (3500 g, 10 min). In all, 5 µL supernatants were loaded onto the Phast Gel Homogenous 20 gel (GE Healthcare, Sweden) and were run in a Phast system (Pharmacia, Sweden) at 240 V. The protein bands were stained with Coomassie blue R 350 (Pharmacia, Sweden).

4.5.4. Plasmin activity measurement and analysis

In study I, PL activity and PG-derived activity were measured from filtration samples with a modified method of Korycka-Dahl et al. (1983). A more detailed description is presented in study I. The inhibitor activities of components against PL activity were evaluated using the linear regression of Michaelis-Menten kinetics (6).

$$\frac{1}{V} = \left(\frac{1}{K'} + \frac{K_m}{K_I S} \right) * \frac{I}{V_{max}} + \frac{1}{V_{max}} + \frac{K_m}{S V_{max}} \qquad (6)$$

where V is the initial velocity, V_{Max} the maximum velocity, K_m the substrate concentration at half-V_{Max}, I the concentration of inhibitor, K'_I the inhibitory constant for uncompetitive inhibition and K_I the inhibitory constant for competitive inhibition.

4.5.5. Lactate dehydrogenase activity measurement

LDH-activity was measured from control cheeses and cheeses made from FC retentate in study V. Cheese samples were pretreated according to the modified method of

Wilkinson et al. (1994). LDH-activity was analyzed from pretreated samples with the method of Wittenberg & Angelo (1979).

4.5.6. Viscosity measurement and analysis

Viscosity was measured using a Physica MCR301- rotation rheometer (Anton Paar, Ostfildern, Germany). A temperature controlled concentric cylinder geometry (CC27) was used for measurements. A more detailed description is presented in study III. Viscosity results were considered by comparing the viscosity results of different samples at shear rates of 10, 100 and 1000 s^{-1} and by fitting the results to the power law model (7) by linearization according to (8)

$$\eta = \eta_0 \gamma^{n-1} \tag{7}$$

$$\log(\eta) = (n-1)\log(\gamma) + \log(\eta_0), \tag{8}$$

where η is the viscosity, η_0 the consistency coefficient, γ the shear rate and n the flow behavior index (Zlokanik and Austria, 2005).

4.5.7. Statistical analyses

Statistical differences were calculated using the analysis of variance (ANOVA)-test. Linear least-square regression was used to find the correlations between components. Statistical analyses were performed using Excel software (Excel version 2007, Microsoft, Redmond, USA).

5. Results

5.1. Plasmin activity during whey protein removal (I)

The retentions of components were measured after milk concentration and after DF steps (Table 7). After the milk concentration step, the retentions of casein, fat, PL and PG were the highest. After DF steps the retention of PL was significantly higher than that of any other components. It was obvious that PL was activated during filtration, because the concentration of the component cannot be increased more than CF.

Table 7. The retention of components after milk concentration (CF 4) and diafiltration steps and the statistical significance of differences between different components (n=4).

		Statistical significance of difference (p-value)					
Components	Retention	Protein	Casein	Fat	Whey protein	Plasmin	Plasminogen
After milk concentration							
Protein	3.53	-	0.009	0.011	0.000	0.060	0.006
Casein	4.15	**	-	0.100	0.00	0.300	0.660
Fat	4.54	*	NS	-	0.003	0.900	0.086
Whey protein	2.15	**	***	**	-	0.003	0.000
Plasmin	4.61	NS	NS	NS	**	-	0.230
Plasminogen	3.95	**	NS	NS	***	NS	-
After diafiltration steps							
Protein	2.83	-	0.001	0.000	0.000	0.001	0.120
Casein	3.39	***	-	0.061	0.000	0.001	0.270
Fat	3.87	**	NS	-	0.000	0.003	0.034
Whey protein	0.63	***	***	***	-	0.000	0.000
Plasmin	7.46	***	**	**	***	-	0.001
Plasminogen	3.16	NS	NS	*	***	***	-

Significance level: * 5%; ** 1%: *** 0.1%; NS not significant

Increase in PL activity was observed when the concentration of β-LG decreased, as shown in Figure 8. However, during DF steps the contents of other components also decreased and therefore the inhibitor component may also be some other component. More detailed inhibitor screening was made using the linearization of Michaelis-Menten kinetics (Table 8). It was obvious that the strongest correlation was obtained by using β-LG as an inhibitor. However, α-LA also showed a strong inhibitory activity against PL.

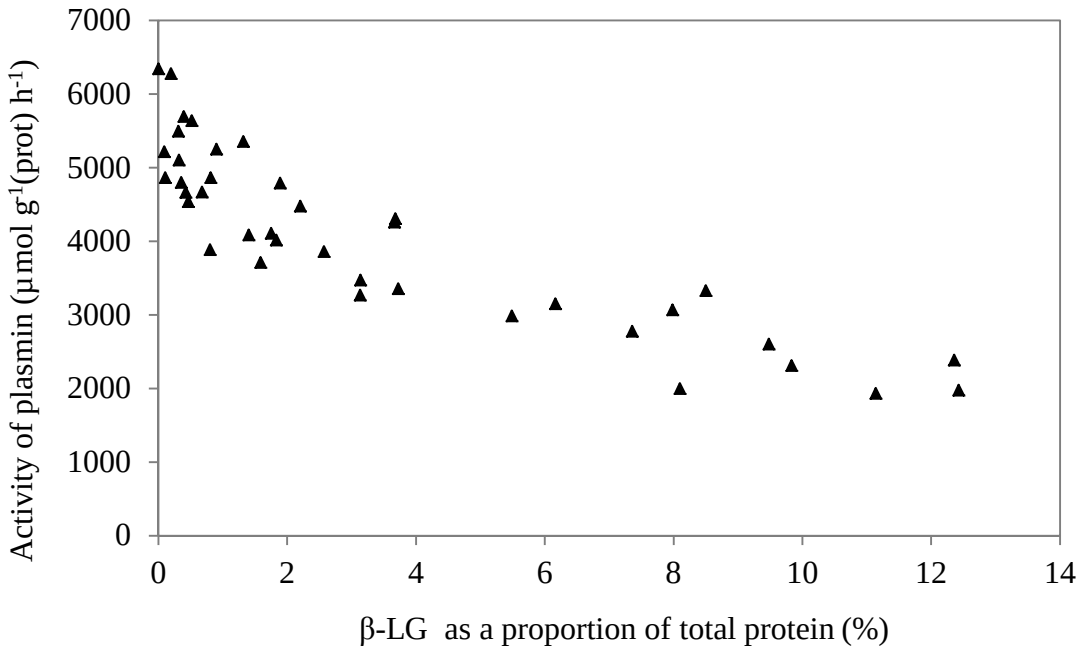

Figure 8. Activity of plasmin as a function of β-lactoglobulin (β-LG) expressed as a proportion of total protein. Results are from four separate filtrations.

Table 8. Linear regression between plasmin activity and the content of possible inhibitor component (n=4).

	1/Plasmin	
Component	R^2	P-value
β-LG	0.926	<0.001
α-LA	0.867	<0.001
Whey protein	0.874	<0.001
Lactose	0.758	<0.05
NaCl	-0.173	0.35

Whey protein-free retentate was pasteurized at 95°C for 15 s. The heat treatment of retentate did not affect the PL activity or PG-derived activity as shown in Table 9.

Table 9. Plasmin (PL) and plasminogen-derived (PG) activity before and after pasteurization at 95°C for 15 s (n=3).

	Activity (μmol g(prot)$^{-1}$ h^{-1})	
	Before pasteurization	After pasteurization
PL	5400 ± 900	4300 ± 700
PG	2000 ± 300	3400 ± 900
PL+PG	7400 ± 1000	7700 ± 1700

5.2. Standardization of calcium content of retentate (II)

Acidification of whey protein-free retentate before MF steps did not affect permeation of the basic component of retentate, as shown in Table 10. Only calcium ions pass through the membrane more efficiently during filtration of acidified retentate than during filtration of non-acidified retentate (Table 10).

Table 10. Permeation of milk components during filtration of acidified and non-acidified retentates (n=3).

Component	Permeation (%)		Statistical significance of the difference
	Non-acidified retentate	Acidified retentate	P-value
Total solids	3.6 ± 0.1	3.5 ± 0.2	0.49
Protein	0.7 ± 0.1	0.9 ± 0.2	0.37
Fat	0.0 ± 0.0	0.0 ± 0.0	1.00
Lactose	130.0 ± 18.0	130.0 ± 36.0	0.94
Calcium	1.9 ± 0.4	7.8 ± 1.7	0.005

The calcium-protein ratio was evaluated during filtrations (Figure 9) and only slight changes in calcium-protein ratios were observed when non-acidified retentates were filtered. However, the calcium-protein ratio decreased linearly during filtration of acidified retentate.

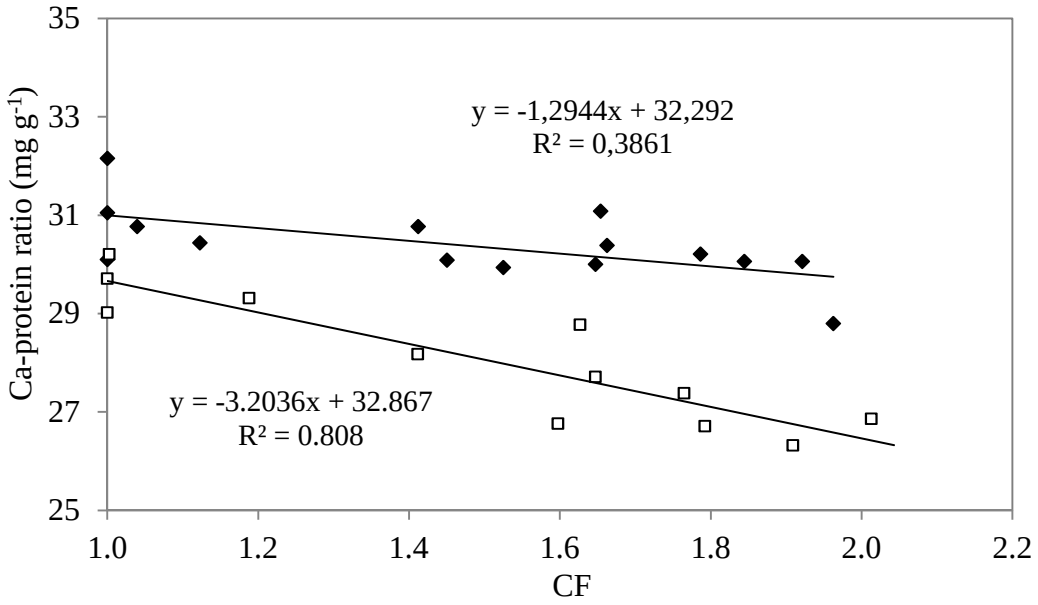

Figure 9. Calcium-protein ratio as a function of concentration factor (CF) during filtration of acidified (□) and non-acidified (♦) retentates (n=3).

5.3. Reducing viscosity of pre-cheese retentate (III)

The viscosity of retentates increased as the total solids content increased during filtration (Figure 10). However, it was obvious that viscosity increase was more efficient during concentration of non-acidified retentate than acidified retentate (Figure 10).

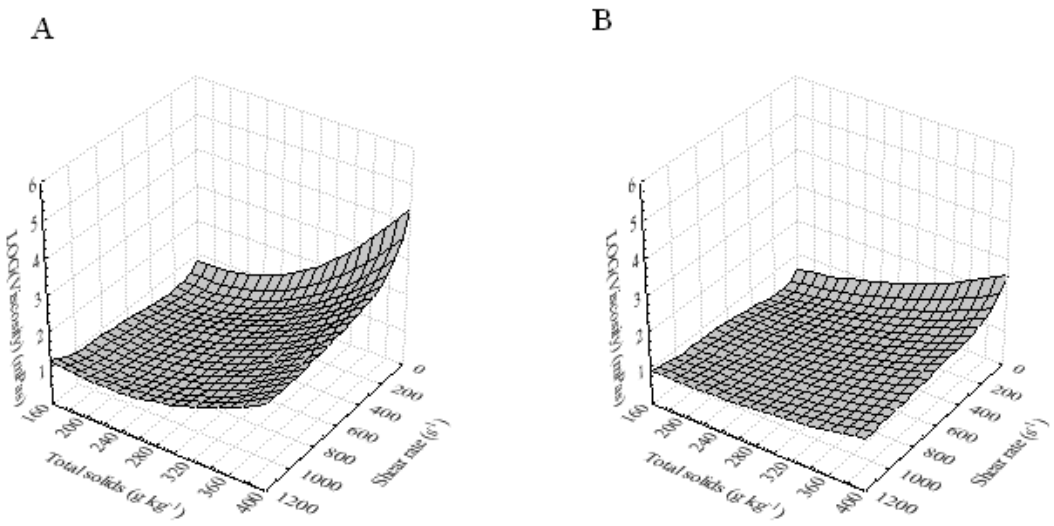

Figure 10. Viscosity of non-acidified (A) and acidified (B) retentate at different shear rates and total solids during filtration (n=434).

A more precise examination was performed by evaluating the flow behavior index and the constant coefficient during filtration of acidified and non-acidified retentate (Table 11). The flow behavior index decreased during concentration of retentate and the constant coefficient increased. There was a statistical difference between acidified and non-acidified retentate already before concentration (total solids content 200 g kg^{-1}) and the difference increased during concentration, as shown in Table 11. The effect of the microbiological acidification on proteolysis was evaluated by SDS-PAGE analysis

(Figure 11). No major differences in protein profile were observed between acidified and non-acidified retentates.

Table 11. Flow behavior index and consistency coefficient of acidified and non-acidified retentates during filtration (n=3).

	Flow behavior index		Consistency coefficient ($mPas^n$)	
Total solids ($g\ kg^{-1}$)	200	300	200	300
Acidified	0.90 ± 0.05	0.76 ± 0.04	1.8 ± 0.2	5.6 ± 0.7
Non-acidified	0.79 ± 0.10	0.59 ± 0.10	2.5 ± 0.4	15.0 ± 5.0
Difference (%)	14	34	28	63
P-value	<0.01	<0.01	<0.001	<0.01

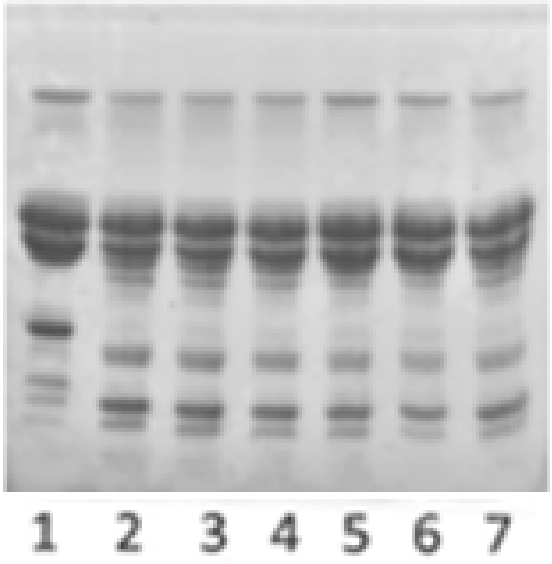

Figure 11. Protein profile (SDS-PAGE) of a sample of non-acidified retentate (lanes 2-4) and acidified retentate (lanes 5-7). Skim milk was used as molecular weight standard (lane 1).

Acidification of the concentrated retentate (total solids 370 $g\ kg^{-1}$) showed that viscosity reduction was most efficient with small pH decreases (Figure 12). However, lower pH values also reduced viscosity significantly (Table 12).

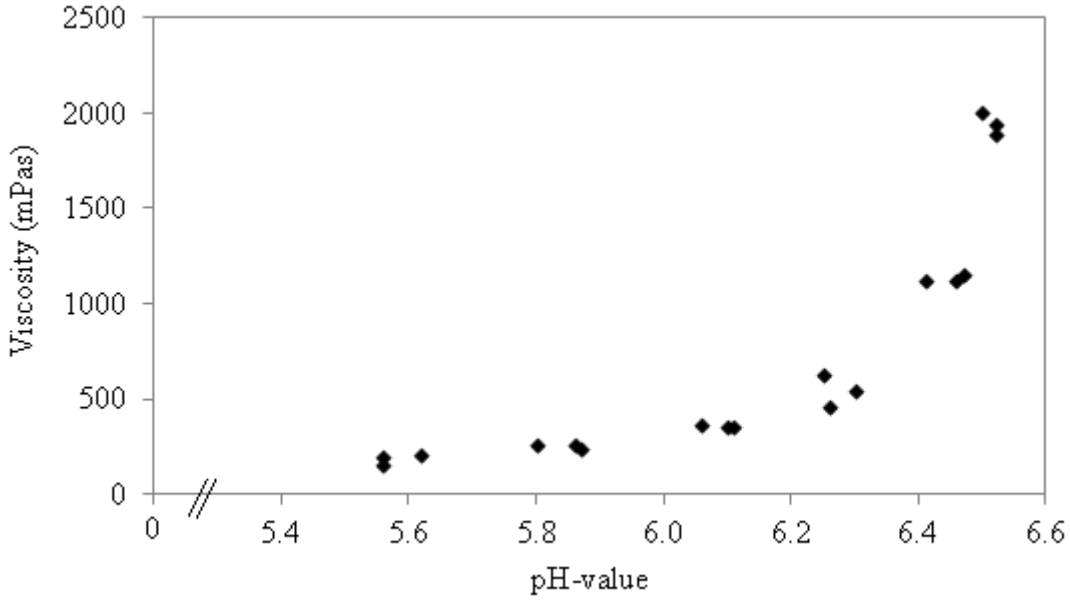

Figure 12. Viscosity of non-acidified and concentrated (total solids 372 g kg^{-1}) MF-retentate at different pH-values during microbiological acidification at 33°C and with a shear rate of 100 s^{-1}. Results are from three separate acidifications.

Table 12. Viscosity of retentate (total solids 370 g kg^{-1}) at different pH values, shear rate 10 s^{-1} and statistical significance of viscosity differences between different pH values (n=3).

		Decrease (%) / Statistical significance			
pH	Viscosity (mPas)	pH 6.5	pH 6.3	pH 6.1	pH 5.6
6.5	14200 ± 400	-	***	***	***
6.3	2500 ± 500	83	-	*	**
6.1	1440 ± 90	90	42	-	***
5.6	600 ± 100	96	76	58	-

[a]Significant level: *: 5%, **:1%, ***: 0.1%

During evaporation of retentates with different pH values, viscosity increased exponentially as a function of total solids (Figure 13) and the correlations were strong. However, the lower the pH value the slower was the viscosity increase during evaporation.

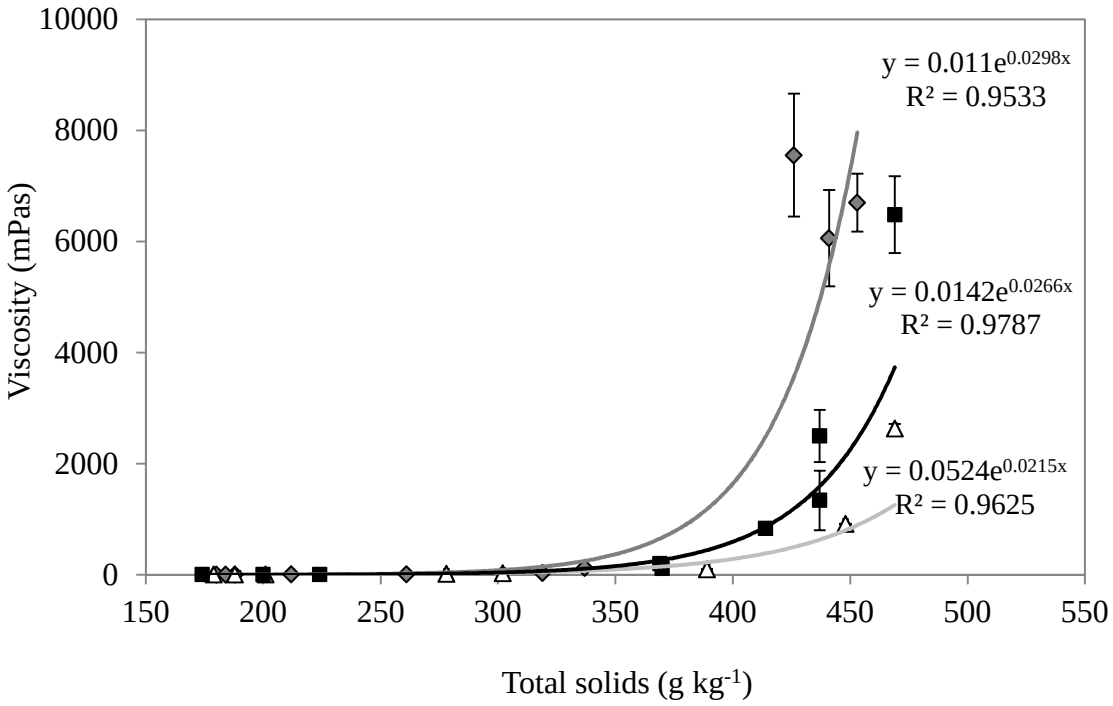

Figure 13. Viscosity of retentates as a function of total solids at pH 6.5 (◊, grey), 6.1 (□, black) and 5.8 (Δ, light grey) during evaporation and with a shear rate of 10 s^{-1}. Results are from three separate evaporations at each pH.

5.4. Effect of acidification on filtration permeate flux

The permeate flux values were measured during filtration of non-acidified and acidified retentates. The permeate flux decreased linearly when the total solids content of retentates increased, as shown in Figure 14. When the flux reduction was compared during filtration of acidified and non-acidified retentate, no significant differences were observed (P = 0.4).

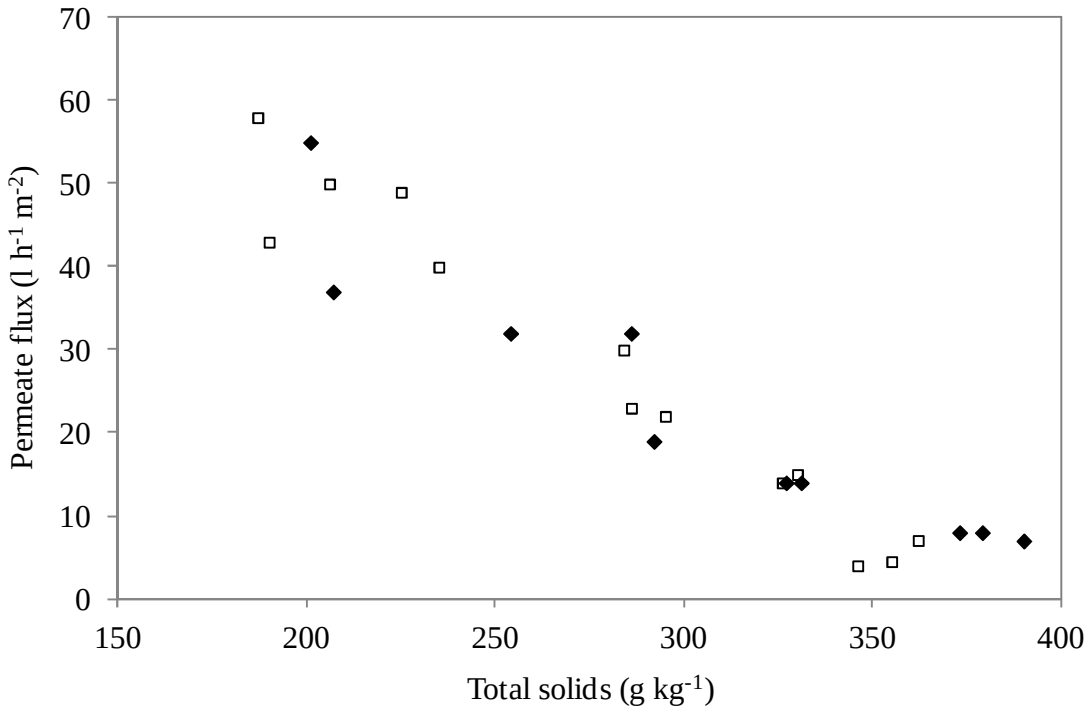

Figure 14. Permeate flux as a function of total solids content during filtration of acidified (□) and non-acidified retentate (♦). Results are from three separate filtrations.

5.5. Effect of peptidase addition on FC cheese ripening (IV)

The proteolysis of cheeses was evaluated by measuring the NPN content of TN during ripening. It was found that the proportion of NPN did not differ significantly between the cheeses with and without peptidase addition (Table 13). Secondary proteolysis of cheeses was evaluated on the basis of FAA content (Table 13). FAA were not observed during ripening of FC cheese, but added peptidase increased FAA at the end of ripening.

No differences were observed in the lactic acid or acetic acid contents between cheeses with and without peptidase addition (data not shown). However, it was found that the

D-isomer of lactic acid was higher in cheeses with added peptidases than in cheeses without added peptidases during ripening (Figure 15).

Table 13. Non-protein nitrogen (NPN) contents of total nitrogen (TN) and free amino acid (FAA) contents during cheese ripening. Results with different letters differ statistically ($p<0.01$) (n=3).

Cheese	Age (day)	NPN/TN (g kg^{-1})	FAA (mmol kg^{-1})
Control	1	2.0 ± 1.0^{a}	ND^{a}
Peptidase addition	1	2.0 ± 1.0^{a}	ND^{a}
Control	28	7.0 ± 1.0^{b}	ND^{b}
Peptidase addition	28	6.4 ± 0.8^{b}	ND^{b}
Control	56	9.0 ± 2.0^{c}	ND^{a}
Peptidase addition	56	9.0 ± 2.0^{c}	42 ± 2^{b}

ND: not detected

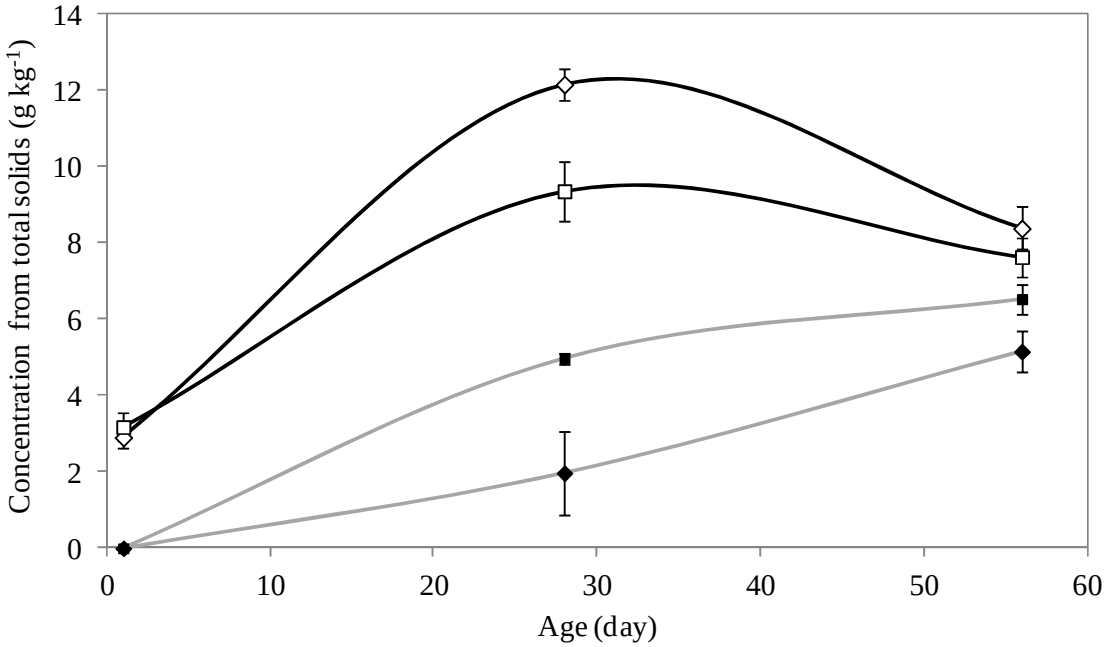

Figure 15. L-lactic acid (black line, white symbol) and D-lactic acid (grey line, black symbol) contents of total solids during ripening of cheeses without (◊) and with added peptidases (□). Results shown are means of three separate studies with standard deviation (I---I).

5.1. Effect of $CaCl_2$ addition on viability of lactic acid bacteria (V)

Chemical contents of cheeses before ripening are shown in Table 14. There were no significant differences in basic chemical contents and only salt contents and calcium-protein ratio were different between FC cheeses made without or with $CaCl_2$ addition. Added $CaCl_2$ decreased the pH value and increased starter growth during ripening, as shown in Table 15. Significant differences were observed in starter lysis when the lysis behavior of LAB was decreased in cheeses with $CaCl_2$ addition (Table 15).

Table 14. Chemical contents of cheeses before ripening. Results in the same row with different letters differ statistically ($p<0.01$) (n=3).

Component	FC cheese without $CaCl_2$ addition	FC cheese with $CaCl_2$ addition
Protein (g kg^{-1})	186.0 ± 3.0^a	187.0 ± 3.0^a
Fat (g kg^{-1})	255.0 ± 3.0^a	261.0 ± 2.0^a
Total solids (g kg^{-1})	481.0 ± 5.0^a	483.0 ± 2.0^a
Lactose (g kg^{-1})	7.8 ± 0.2^a	7.4 ± 0.4^a
Lactic acid (g kg^{-1})	1.3 ± 0.1^a	1.3 ± 0.1^a
Salt (g kg^{-1})	15.0 ± 0.0^a	19.0 ± 1.0^b
Ca:protein (mg g^{-1})	20.6 ± 0.0^a	31.0 ± 0.8^b

Table 15. Value of pH, content of lactic acid bacteria (LAB) and activity of lactate dehydrogenase (LDH) during cheese ripening. Results in the same column with different letters differ statistically ($p<0.01$) (n=3).

Cheese	Age (day)	pH	LAB log(cfu g^{-1})	LDH-activity (U g^{-1})
Control	1	6.0 ± 0.0^a	4.6 ± 0.1^a	0.00 ± 0.00
$CaCl_2$ addition	1	5.8 ± 0.0^b	5.3 ± 0.1^b	0.00 ± 0.00
Control	28	5.4 ± 0.0^c	6.4 ± 0.1^c	0.40 ± 0.10^a
$CaCl_2$ addition	28	5.3 ± 0.0^d	8.6 ± 0.2^d	0.03 ± 0.00^b
Control	56	5.4 ± 0.0^c	6.4 ± 0.2^c	4.40 ± 0.50^c
$CaCl_2$ addition	56	5.1 ± 0.1^e	8.7 ± 0.2^d	0.04 ± 0.02^b

6. Discussion

6.1. Plasmin activity during whey protein removal (I)

Concentration of milk with MF concentrated PL, PG, casein and fat. It is well known that PL and PG are part of casein micelles when milk pH is higher than 4.6 (Korycka-Dahl et al., 1983; Politis et al., 1992) and the result was therefore expected. However, during DF the PL activity increased and it was obvious that PL was activated as a result of whey protein removal. The inhibitor activities of components against PL were evaluated by linearization of the Michaelis-Menten kinetics. It was clear that β-LG showed the strongest inhibitory activity against PL. However, the inhibitory activity of α-LA was also strong and it was not possible to verify the inhibition of β-LG because the α-LA and β-LG contents decreased in parallel during filtration (Heino, 2009). These results are in agreement with those of Rollema and Poll (1986), who showed that β-LG inhibited PL activity.

Pasteurization of retentate at 95^{o}C for 15 s did not affect the activities of PL or PG-derived. However, it is well known that thermal inactivation of PL at 90^{o}C in milk is significant (Benfeld et al., 1997; Parado et al., 2007; Saint Denis et al., 2001). During milk heat treatment, PL is inactivated when the free thiol groups from unfolded β-LG or α-LA bind with the serine-rich structure of PL (Rollema & Poll, 1986; Saint Denis et al., 2001), thus giving rise to the different heat stabilities of PL in whey protein-free retentate and in milk. However, more research is needed to evaluate the heat stability of PL in longer heat treatments because the final concentration of milk is with evaporation.

In cheese-making, PL initiates the primary proteolysis of cheese with added coagulant. It is known that high PL activity decreases ripening time and increases cheese flavor formation (Barrett et al., 1999; Bastian et al., 1997; Farkye & Fox, 1992). However, whey proteins are also removed in traditional cheese-making, probably causing activation of PL. The negative effect of high PL activity before cheese-making is the reduced cheese yield and recovery of protein (Mara et al., 1998; Srinvasan & Lucey, 2002). PL-hydrolyzed milk also has weaker coagulation properties than those of traditional milk (Srinvasan & Lucey, 2002). During development of the FC cheese-making process, the PL activation must be handled by minimizing the processing time of whey protein-free retentate so that the hydrolysis of protein is insignificant before the start of actual cheese-making.

6.2. Standardization of calcium content of retentate (II)

During acidification of retentate, the solubility of calcium phosphate from casein micelles increases and therefore the permeability of calcium is higher in acidified retentate than in non-acidified retentate during filtration, which is in agreement with previous findings (Brandsma & Rizvi, 1999; Salvatore et al., 2011). CF did not affect the calcium-protein ratio when non-acidified retentate was filtrated. However, the calcium-protein ratio linearly correlated with CF when acidified retentate was filtered, which makes it possible to standardize the ratio. The calcium-protein ratio must be standardized before making FC cheese because a too high ratio increases whey release during ripening, which negatively affects the texture (Pastorino et al., 2003). The calcium-protein ratio of hard cheeses (Edam, Cheddar, Gouda and Emmental) is 29 mg g^{-1} or higher (Lucey & Fox, 1993) and the CF can be low. However, in soft cheeses the calcium-protein ratio is below 20 mg g^{-1} (Lucey & Fox, 1993) and CF must be high or a

DF step can be used to reach this ratio. During standardization of calcium content, the conditions should be standardized because both pH and temperature greatly affect the solubility of calcium ions (Lucey & Fox, 1993). Lower pH and temperature values increase the solubility of calcium and permeation of calcium (Kaombe et al., 2012).

6.3. Reducing the viscosity of pre-cheese retentate (III)

Viscosity of retentate increased during concentration, in agreement with previous findings (Vadi & Rizvi, 2001; Velez-Ruiz & Barbosa-Canovas, 1998). However, the flow behavior index of the retentates was below 1 already before the concentration (total solids 200 g kg^{-1}) and therefore the retentates were non-Newtonian. Previous studies have shown that concentrated whole milk is Newtonian (flow behavior index is 1) below 220-300 g kg^{-1} total solids contents (Velez-Ruiz & Barbosa-Canovas, 1998, 2000). However, in this study the whey protein-free milk concentrate was used and the concentration of the casein micelles was high. It is well known that large aggregates such as casein micelles increase viscosity (de Kruif, 1997).

It was obvious that acidification of the retentate reduced viscosity and the increase of viscosity during concentration. Proteolysis in non-acidified and acidified retentate was at the same level, and therefore the reason for the viscosity difference is not linked to proteolysis. However, it is known that acidification of milk increases the solubility of calcium phosphate from casein micelles (Fox & McSweeney, 1998). Liu and Guo (2008) also found that the size of the casein micelle decreases with acidification and that the structure of the casein micelle is most compact at pH 5.5. Viscosity can be related to the volume fraction of components by the Eilers equation (9) (Snoeren et al., 1982)

$$\frac{\eta}{\eta_{ref}} = \left[1 + \frac{1.25\phi}{1 - \phi/\phi_{\max}}\right], \qquad (9)$$

where η/η_{ref} is the relative viscosity, ϕ the volume fraction = V*C, where V is the volume of component and C the concentration, and $\phi_{\max}$ is the maximum volume fraction, which is constant for the same types of systems. Thus, if the volume of micelles is smaller, then the overall viscosity will not increase with increasing concentration of normal micelles. The most probable reason for viscosity reduction is the reduced casein micelle size during acidification. These observations are essential when cheese is made from FC retentate.

6.4. Effect of acidification on filtration permeate flux

Acidification of retentate before filtration did not affect the flux which disagrees with the previous findings of Brandsma and Rizvi (1999), who reported that the acidification of milk retentate before filtration reduced the flux below that of non-acidified retentate in the same filtration conditions as in this work. However, whey proteins greatly influence membrane fouling during filtration (Marshall et al., 1997) and they were included in the retentates or milks used as feed in the previous studies (Brandsma & Rizvi, 1999; Eckiner & Zottola, 1992; Ernstrom et al., 1980). Membrane fouling begins when whey proteins form a layer on the membrane surface. Free calcium ions are able to bind to the whey proteins in addition to the casein micelles (Vetier et al., 1988). This fouling is efficient and has a significant impact on the permeate flux (Brandsma & Rizvi, 1999; Eckiner & Zottola, 1992; Ernstrom et al., 1980; St-Gelais et al., 1992a). However, whey protein-free retentate was unable to form the first fouling layer, and the acidification or free calcium ions did not decreased the permeate flux value. During

acidification, viscosity reduction also increases the filtration flux according to equation (10).

$$J = \frac{\Delta P}{\eta R}, \qquad (10)$$

where J is the flux, ΔP the pressure difference between the retentate and permeate sides, η the viscosity and R the resistance factor which depends on membrane material, concentration polarization and cake layer on the surface of the membrane (Makardij et al., 2010).

6.5. Effect of peptidase addition on FC cheese ripening (IV)

NPN did not differ significantly between the cheeses with and without peptidase addition, which indicates that peptidase did not affect primary proteolysis. Secondary proteolysis of cheeses was evaluated with FAA content; these components were not found in FC, which can be explained by delayed lysis of starter made from concentrated milk, as shown previously (Hannon et al. 2006; Saboya et al. 2001). In this study, the added peptidases increased the content of FAA at the end of ripening, indicating that ripening of FC cheese can be accelerated by peptidase addition. The FC process makes it possible to add enzymes to cheese without losses to whey and therefore it is possible to obtain a better effect than in traditional cheese-making processes even with low dosage.

No differences were found in the lactic acid or acetic acid contents between cheeses with and without peptidase addition. However, added peptidase enzyme significantly

affected the lactose fermentation of LAB. It was found that the D-isomer of the lactic acid content was higher in cheeses with added peptidases than in cheese without added peptidases during ripening. This may be due to the higher growth rate of *Leuc. mesenteroides,* which produces D-lactic acid via the phosphoketolase pathway according to equation (2). In Dutch-type cheese, lactic acid is mainly produced by the glycolytic pathway of *Lactococcus lactis* subsp *lactis* and *Lactococcus lactis* subsp *cremoris* according to equation (1) (Fox and McSweeney 1998). It is well known that *Leuc. mesenteroides* grows poorly and slowly in milk (Server-Busson *et al.* 1999), because most *Leuc. mesenteroides* strains are unable to degrade and utilize casein (Bellengier et al. 1997). However, it is known that increased NPN contents and proteolytically active *L. lactis* strains increase the *Leuc. mesenteroides* growth rate in milk (Foucaud et al. 1998), and it can be assumed that added peptidases promoted the growth of *Leuc. mesenteroides*. Such changes in microbiological composition should be considered during cheese production with different enzyme additions.

6.6. Effect of $CaCl_2$ addition on viability of lactic acid bacteria (V)

$CaCl_2$ addition affected the calcium-protein ratio of cheeses as planned. However, there was also a difference in salt content, which was measured with potentiometric titration of chloride and calculated according to chloride ions.

It was obvious that added $CaCl_2$ decreased pH because H^+ ions are released when additional complexes between calcium and phosphates are formed (Fox et al., 2004). Decreased pH did not inhibit starter growth, and moreover LAB growth increased during addition of $CaCl_2$ and decreased pH. It was also obvious that lysis of LAB decreased when $CaCl_2$ solution was added, which may explain the increased LAB

content of cheese made with $CaCl_2$ addition. The observation agrees with the results of Lortal et al. (1991) who found that $CaCl_2$ addition decreased lysis of *Lb. helveticus* in liquid medium.

In previous studies, it has been assumed that the reason for decreased lysis is high nutrition content or buffering capacity in cheeses made from concentrated milk (Hannon et al., 2006; McMahon, et al., 1997; St-Gelais et al., 1992). However, it was shown that decreased pH does not increase the lysis of LAB, and moreover that it is the content of calcium which decreases lysis. When cheeses are made from concentrated milk, pH decrease increases the soluble calcium content in milk and may affect the starter behavior. Calcium content can be reduced by acidification and filtration steps, as previously shown in the section on calcium standardization.

7. Conclusions

Cheese-making from FC milk retentate does not include traditional curd cutting and whey draining after coagulation. Milk is concentrated to the final total solids content of cheese with filtration and evaporation steps, and the FC process consists of whey protein removal and standardization of lactose and calcium contents with MF. During whey protein removal, it was observed that indigenous milk PL enzyme was activated, and that the heat stability of PL increased when whey proteins were removed. Attention should be paid to this activation and to the heat stability of PL during FC process development, because too early proteolysis may reduce the cheese yield and affect the coagulation properties of retentate. A continuous process is therefore essential when cheeses are made with the FC process. The calcium content of cheese greatly affects its final structure; basically the higher the calcium-protein ratio the harder is the structure of cheese. Calcium content can be standardized by acidification and filtration steps and the CF affects the final calcium-protein ratio.

Processability of FC retentate is essential during cheese-making with the FC process. It is well known that milk concentration increases the viscosity of milk, which further complicates processing. However, it was shown that acidification of whey protein-free retentate reduced the viscosity and the viscosity increase during concentration by filtration and evaporation. The reason for the viscosity reduction may be the reduced size of casein micelles as a consequence of increased solubility of colloidal calcium phosphates during acidification. The acidification step is essential during cheese-making from FC retentate. Earlier it has been shown that acidification of milk decreases the filtration flux, although acidification of whey protein-free retentate did not affect its

further processing. It can be assumed that whey protein greatly affects the fouling of filtration membranes at low pH values.

In previous studies, delayed lysis of starter has been demonstrated in cheese made from concentrated milk. To improve the lag of peptidase activity a study was made of, the effect of peptidase addition on cheese made from FC retentate. Added peptidases did not affect the NPN contents during cheese ripening, and therefore it can be assumed that there were no significant differences in primary proteolysis and bitter flavor formation. FAA was not detected at the end of ripening of FC cheese, indicating that secondary proteolysis was at a low level. However, FAA content can be increased by addition of peptidases and therefore it is possible to increase the secondary proteolysis of cheese. In the FC cheese-making process, enzymes can be used to decrease the ripening time because no traditional whey draining is performed and there are no enzyme activity losses to whey. However, added peptidases changed the lactose fermentation during ripening by increasing the formation of D-lactic acid in FC, which may explain the higher growth rates of *Leuc. mesenteroides* strains. Such changes in microbiological composition should be considered during the development of cheese-making processes including enzyme addition.

The FC process is a suitable process for studying how different components affect cheese ripening. $CaCl_2$ addition affected ripening, and it was clear that added calcium increased the growth and decreased the lysis of LAB. According to this observation it appears that it is essential to standardize calcium content before cheese-making, and that the buffering capacity is not the main reason for delayed lysis of LAB in cheeses made from concentrated milk.

8. References

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